CAROTINOIDS AND RELATED PIGMENTS

THE CHROMOLIPOIDS

BY

LEROY S. PALMER, Ph.D.

PROFESSOR OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA

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GENERAL INTRODUCTION

American Chemical Society Series of Scientific and Technologic Monographs

By arrangement with the Interallied Conference of Pure and Applied Chemistry, which met in London and Brussels in July, 1919, the American Chemical Society was to undertake the production and publication of Scientific and Technologic Monographs on chemical subjects. At the same time it was agreed that the National Research Council, in coöperation with the American Chemical Society and the American Physical Society, should undertake the production and publication of Critical Tables of Chemical and Physical Constants. The American Chemical Society and the National Research Council mutually agreed to care for these two fields of chemical development. The American Chemical Society named as Trustees, to make the necessary arrangements for the publication of the monographs, Charles L. Parsons, Secretary of the American Chemical Society, Washington, D. C.; John E. Teeple, Treasurer of the American Chemical Society, New York City; and Professor Gellert Alleman of Swarthmore College. The Trustees have arranged for the publication of the American Chemical Society series of (a) Scientific and (b) Technologic Monographs by the Chemical Catalog Company of New York City.

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The development of knowledge in all branches of science, and especially in chemistry, has been so rapid during the last fifty years and the fields covered by this development have been so varied that it is difficult for any individual to keep in touch with the progress in

branches of science outside his own specialty. In spite of the faci
for the examination of the literature given by Chemical Abstracts
such compendia as Beilstein's Handbuch der Organischen Ch
Richter's Lexikon, Ostwald's Lehrbuch der Allgemeinen Ch
Abegg's and Gmelin-Kraut's Handbuch der Anorganischen Cl
and the English and French Dictionaries of Chemistry, it often
a great deal of time to coördinate the knowledge available up
single topic. Consequently when men who have spent years i
study of important subjects are willing to coördinate their know
and present it in concise, readable form, they perform a service o
highest value to their fellow chemists.

It was with a clear recognition of the usefulness of reviev
this character that a Committee of the American Chemical So
recommended the publication of the two series of monographs u
the auspices of the Society.

Two rather distinct purposes are to be served by these monogr
The first purpose, whose fulfilment will probably render to che
in general the most important service, is to present the know
available upon the chosen topic in a readable form, intelligib
those whose activities may be along a wholly different line. M
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nected with other work which on the surface appears far afield
their own. These monographs will enable such men to form c
contact with the work of chemists in other lines of research.
second purpose is to promote research in the branch of science co
by the monograph, by furnishing a well digested survey of the p
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investigation needs to be extended. To facilitate the attainme
this purpose, it is intended to include extended references to the l
ture, which will enable anyone interested to follow up the subje
more detail. If the literature is so voluminous that a complete
ography is impracticable, a critical selection will be made of
papers which are most important.

The publication of these books marks a distinct departure i
policy of the American Chemical Society inasmuch as it is a se
attempt to found an American chemical literature without pri
regard to commercial considerations. The success of the ventur
depend in large part upon the measure of coöperation which ca
secured in the preparation of books dealing adequately with top

general interest; it is earnestly hoped, therefore, that every member of the various organizations in the chemical and allied industries will recognize the importance of the enterprise and take sufficient interest to justify it.

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PREFACE

Color in nature may properly be divided into two groups, namely, color due to structure, which is caused by light reflections from colloidal particles of air or water, and color due to pigments, which is caused by substances having remarkable powers of absorption of light rays of certain wave length and reflection of others. The reflected rays, of course, give the pigment its color.

The present monograph treats of pigmented substances having a yellow, yellow-orange, orange, red-orange and red color. So far as the author is aware no authentic instances of structural colors of these hues have been reported. In fact, the wave length of light in these regions of the spectrum is probably too great for such a phenomenon to occur for colloidal emulsions having the refractive index of air or water. The particular pigments to be considered are widely distributed in every stage of living matter, and are perhaps more frequently encountered than any other class of natural pigments. They have attracted the attention of the biologists for at least 100 years. Among the earliest inquiries were those of Caventau (1817) and Goebel (1823). The former was interested in the yellow pigment of the daffodil, the latter in the pigments of the crab and in the feet of doves and geese.

Active investigation of these pigments in plants and animals has been confined to the past fifty years. It has only been within the past fifteen years, however, that the chemical composition of any of these pigments has been definitely established. Their constitution still offers a fascinating problem for the organic chemist.

The writer favors Tswett's terminology of carotinoids for these pigments. From the standpoint of phytochemistry there is definite evidence for the existence of five carotinoids, with indications that several others also occur. When it was discovered that certain of the carotinoids occur in animals it was believed that both plants and animals synthesize these pigments. It soon became apparent, however, that the chromolipoids found in the higher animals, at least those

which have been identified as carotinoids, are in reality merely derived from the food. The assumption therefore seems justified that a similar biological relationship exists between all the chromolipoids of plant and animal life; in other words, that all animal chromolipoids are derived pigments and are either true or modified carotinoids.

The writer has had three main ideas in mind in preparing this monograph. First, he has attempted to compile a thorough history of the development of the chemistry of the plant and animal chromolipoids. This has not been attempted before in this particular field. Second, he has tried to present such information regarding the pigments as would be useful to workers who desire to attack the many interesting problems in this branch of plant and animal chromatology. Third, he has made an effort to point out lines of research which might prove attractive to those interested in this subject. The author hopes that he has had a reasonable measure of success in his efforts.

For the convenience of readers who have not been trained in systematic nomenclature the scientific name of the individual species of plants and animals in which carotinoids occur has been supplemented wherever possible by the common name. For the plants, this information has been drawn largely from Bailey's Cyclopedia of Horticulture.

It may be of interest to the reader to know that the carotinoid pigments in plants and animals have proved to be of some practical importance. The uses to which their occurrence in animals have been put are reserved for discussion in Chapter XI of the monograph. The occurrence of carotinoids in plants, particularly green plants, formed the basis for the construction of the light filters used by the American Army during the late war for the detection of camouflaged foliage. Natural green foliage reflects both green and red light, due to the fact that the chlorophylls and carotinoids are present together in the chloroplastids. The visibility of the rays reflected from the carotinoids is so low in the presence of the chlorophylls which are present in five to six times the concentration of the orange and red pigments, that green color only appears to be reflected. However, it was found possible to construct a light filter which absorbed practically all light rays except a wide band in the red at about 700$\mu\mu$, and a narrow band, with low transmission in the green at about 500$\mu\mu$, so that natural green foliage viewed through this filter appeared red, while camouflaged foliage on which green paint only was used, appeared green.

The writer has encountered so much difference of opinion regarding the correct pronunciation of certain words which are used very fre-

quently in this monograph that he begs to suggest, for the sake of uniformity, the following pronunciations which are believed to be in keeping with the best modern English usage.

Carotin = Kăr′ ō̇-tĭn.
Carotinoid = Kăr″ ō̇-tĭn-oĭd′.
Lipochrome = Lĭp′ ō̇-krōm.
Chromolipoid = Krōm-ō̇-lĭp′ oĭd.
Chromatogram = Krōm″-ătō-grăm′.

In conclusion the author wishes to acknowledge his indebtedness to his colleague, Dr. R. A. Gortner, for many helpful criticisms in the preparation of the manuscript; to Dr. Josephine E. Tilden of the Department of Botany, University of Minnesota, for classifying the algæ in which carotinoids occur, and to Mr. Lloyd A. Jones, of the Eastman Kodak Co., for information regarding the light filters devised in the Eastman Research laboratories during the war.

ST. PAUL, MINN.
July 1, 1922.

CONTENTS

CAROTINOIDS AND RELATED PIGMENTS

Chapter I

General Distribution of Carotinoids. The Pigments Defined

Red, orange and yellow pigments which can be extracted from tissues by fat solvents are found abundantly in all forms of living matter. In the plant world they are present in nearly all species ranging from bacteria, the lowest forms of cryptogams, to the dicotyledons, the highest forms of phanerogams. Similarly in the animal kingdom we find yellow to reddish pigments in all forms of both invertebrates and vertebrates, from protozoa to man. The earliest workers in both the plant and animal fields naturally based the classification of the pigments on simple properties, so that it is not surprising to find that many names have been proposed for what is obviously the same pigment.

This diversity in nomenclature is found to be especially true among the yellow animal pigments, and can be traced in most instances to slight variations in certain of the simple properties which were regarded as specific for various types of pigment. In some cases these variations were due to the fact that the method employed for the isolation of the pigment did not insure its freedom from other pigments of similar but not identical properties. In other cases the variations were due to the examination of the pigment in amorphous condition or in solution, without reference to the possible effect which these states might exert upon the particular properties being studied. Again, there was frequently an abundant contamination with lipoid impurities, which are invariably separated with the pigments from animal tissues. Another, still more important cause for these variations was the failure to protect the pigments from oxidation. The true carotinoids, which unquestionably comprise the great majority of yellow

and red tinted animal pigments included under the older term lipochrome, are characterized by the ease with which they oxidize when in solution or in the solid state. The earlier workers did not recognize, however, that some of the most characteristic properties of these pigments are subject to modification even in the earliest stages of oxidation. This is particularly true of the color reactions with various reagents, and the spectroscopic properties, which have been used so widely, and many times exclusively, as the basis for the classification of the animal chromolipoids.

Confusion in terminology, however, has not been confined to the animal pigments. The chief difficulty regarding the plant carotinoids has been the proposal of names already in use for pigments of obviously different composition and properties. For example, the name xanthophyll, as used by various workers in the field of plant pigments, has been the cause of so much confusion in the nomenclature as to be very disconcerting to many students of this subject.

It is not surprising, therefore, that certain investigators have attempted to bring some semblance of order to the confusion by proposing one name to cover all the names previously proposed for pigments of like or similar properties. A brief history of these attempts with their resulting influence on the nomenclature of plant and animal chromatology may prove of interest at this point.

Luteins

The first attempt to bring various yellow pigments together under one name is found in Thudichum's (1869) classic paper, in which the yellow pigments found in many tissues of both vegetable and animal origin are grouped under the name "luteine," or luteins. The name was obviously suggested by the fact that the characteristic yellow pigment of the corpus luteum on the ovaries of mammals, especially that of the cow, is one of the representatives of the "luteine" pigments. It is doubtful whether Thudichum was familiar with the work of Piccolo and Lieben (1866), who had crystallized the corpus luteum pigment a few years previously and named it luteohämatoidin or hamolutein. However, Thudichum mentioned the work of Holm (1867), who isolated the corpus luteum pigment and called it hämatoidin.

Thudichum's luteins included, besides the corpus luteum pigment, the yellow pigment of blood serum, adipose tissue and butter, and the yellow pigment of egg yolk. The vegetable pigments in the lutein

group included the pigment of yellow maize, and annatto seeds, the pigment of the carrot root and of yellow leaves, such as those of the *Coleus*, and the pigments which characterize the stamens and petals of many flowers.

The basis for the classification of these pigments into one group was: (1) their common solubility in alcohol, ether and chloroform, and in albuminous liquids like blood serum; (2) the fact that their solutions showed three absorption bands in the blue, indigo and violet region of the spectrum; (3) the fact that they could be crystallized in the form of rhombic plates; (4) certain common chemical reactions, such as their precipitation by mercuric acetate and mercuric nitrate, and their blue color reaction with nitric acid, when the pigments were in the solid state or in solution in acetic acid; (6) and their affinity (5) for albumin, as in blood serum and the fluid of ovarian cysts, from which the pigments are extracted with difficulty.

Thudichum's classification never received wide adoption. In fact, the luteins, as defined by Thudichum, comprise a number of different pigments. Moreover, our present knowledge regarding practically all the pigments which were included in this classification shows that certain of the characteristic lutein properties [1] are specific only for certain individuals of the group. The final abandonment of this classification appears in the recent application of the name lutein by Willstätter and Escher (1912) to the specific crystalline pigment isolated by them from the yolks of hen's eggs. This use of the name appears to the author to be illogical both from the standpoint of function and anatomy as well as on other biological grounds. The name lutein obviously suggests the body from which the name was derived, namely, the corpus luteum. The yolk of the egg of the oviparous animal is certainly not related to the corpus luteum either functionally or anatomically. Moreover, the egg yolk pigment has been demonstrated by Palmer (1915) to be physiologically as well as chemically identical with at least one, and probably a group of the plant pigments which are known as xanthophylls. Egg yolk xanthophyll is, in fact, a true carotinoid, or mixture of carotinoids, and no further designation appears necessary.

[1] The heretofore inexplicable property of being precipitated by mercury salts, ascribed to the luteins by Thudichum, becomes clear only in the light of Palmer's (1914 c) observations that the albumin with which carotin is sometimes associated in the blood serum of animals is precipitated by mercury salts. It is also possible that Thudichum observed the phenomenon of the adsorption of carotin by mercury salts described by Tswett (1906 b).

Lipochromes

Krukenberg (1882k, 1886), a number of years after Thudichum, proposed the name lipochrome to cover all the animal and plant pigments which had previously been known as luteins, carotins, zoonerythrin, tetronerythrin, chlorophan, xanthophan and rhodophan. The name lipochrome has been widely adopted and due to the very broad basis upon which the name was founded it has been applied to numerous plant and animal pigments not mentioned by Krukenberg, or unknown to him. Krukenberg believed that all the pigments which he proposed to designate as lipochromes were associated with fat in their natural state, and the name suggests this supposition as well as their capability of existing in association with fats and oils.

It was obviously the intention of the originator of the name lipochrome to limit it to pigments of yellow or reddish tints, but the name itself is applicable to pigments of many other colors, such as chlorophyll and many vegetable dyes of various colors, which have a marked affinity for fat. Numerous workers object to the use of the name lipochrome on this account. Kohl (1902a), for example, in his extensive monograph on carotin, objects to designating this pigment as a lipochrome because of the numerous cases in which it is known to occur free from fat, and also because he believes that where carotin is actually found associated with fat it is in combination with the fat and not merely in solution.

The particular properties by which Krukenberg (1886) proposed to judge whether a pigment should be classified as a lipochrome are, in general, as follows: They are soluble in alcohols (methyl, ethyl and amyl), ether, chloroform, benzene, carbon disulfide, petroleum ether and acetone; in the solid state they are colored blue-green to blue by concentrated sulphuric and nitric acids and generally blue-green with iodine in potassium iodide; they show two and sometimes three absorption bands in the blue and violet region of the spectrum; they are not destroyed on boiling with alcoholic caustic alkalies; in the solid state they are greenish-yellow, yellow, orange or red, and their solutions are yellow; they are very sensitive to light and readily bleach, the bleached pigments being similar to cholesterol.

Subsequent investigations of the lipochromes, using the class characteristics defined by Krukenberg, have added very little to our knowledge of the properties of these pigments considered as a group, but have served merely to define more closely certain of the criteria enu-

merated. Krukenberg believed that all lipochromes should be regarded as composed of carbon, hydrogen and oxygen, and free from nitrogen. At the present time hydrocarbons, such as carotin and its isomers, as well as the oxyhydrocarbons, fulfill all the characteristics of lipochromes. Probably wider use has been made of the color reactions with concentrated sulphuric and nitric acids and with iodine in potassium iodide than any of the other class characteristics for identifying pigments as lipochromes although many studies have also included spectroscopic observations. Unfortunately the color reactions and spectroscopic properties are subject to greater variation than any of the others upon which the classification is based. The result of the color tests as well as the quality of the color is often influenced strongly by admixture with foreign substances, and this is apparently especially true for the reaction with iodine in potassium iodide. Similarly the spectroscopic absorption properties are subject to wide variation as to the position of the bands as well as their definiteness by reason of admixture with impurities, concentration of pigment, and the solvent employed.

Lipoxanthins

A more recent attempt than Krukenberg's to bring all the known plant and animal pigments with like properties under one name is that of Schrötter-Kristelli (1895a), who proposed to group together all the various plant and animal coloring matters which had previously been known as etiolin, chlorophyll yellow, xanthin, anthoxanthin, lutein, xanthophyll, chrysophyll, carotin, phylloxanthin, phycoxanthin, erythrophyll, solanorubin, lipoxanthin, haematochrom, chlororufin, bacteriopurpurin, haemolutein, vitellorubin and tetronerythrin. He regarded these pigments as at least an homologous group, if not completely identical, and chose the name lipoxanthin as the most suitable for a general designation. The chief characteristics of the lipoxanthins, according to Schrötter-Kristelli, are their affinity for fats, their insolubility in water, their blue color reaction with concentrated sulphuric acid, their absorption of the violet end of the spectrum, their lack of fluorescence when in solution, and their ease of destruction by light and oxygen. Schrötter-Kristelli believed that the slight differences in the spectroscopic properties of the various pigments were due to their ease of destruction.

According to this author lipoxanthins have been demonstrated to occur in all green leaves, in autumn leaves, in many flowers and fruits,

in arils and roots, in algæ lichens, fungi and bacteria; among animals they have been demonstrated in the egg yolk of the sea-spider, in the retina of bird's eyes, in insects, such as *Chrysomelidæ* and *Coccinellidæ*, and in the secretions of various crustaceæ, such as various kinds of *Diaptoma*, and *Maia squinado* as well as in still lower forms of animal life.

The lipoxanthins are thus seen to be a more or less indefinite group of pigments, whose classification together under one head is secured just as well by the older term lipochrome, which no doubt explains why the proposed term never received wide recognition.

Chromolipoids

As our knowledge of the so-called lipochromes and lipoxanthins has been extended by exhaustive researches regarding the various individual representatives from both plant and animal sources the objections which have been raised by various workers to terms such as lipochrome and lipoxanthin seem to be more and more valid. The botanists have been the first to definitely break away from the old terminology as exemplified by the citation from Kohl's monograph. Czapek (1913a) proposes to meet the objections to the name lipochrome by calling the pigments chromolipoids. His point of view is that the lipochromes, at least in plants, are to be classed with the lipoids by reason of their many fat-like properties, especially solubility, and also because of their widespread occurrence in cells in which lipoids are known to exist. Moreover, the lipochromes, in common with phosphatides and sterols, absorb oxygen very readily. Czapek's terminology has much in its favor, in the opinion of the author. It is at least preferable from many standpoints to the more or less misleading term lipochrome.

Carotinoids

Attempts have not been wanting to secure uniformity in the terminology of the yellow plant pigments. The first yellow plant pigment to be isolated in crystalline form was carotin, the pigment of the root of the cultivated carrot, *Daucus carota*. At one time the name carotin was used to cover all the plant chromolipoids. When it became known that differences existed between many of the so-called carotins, the name was changed to carotinen, or investigators spoke of the

"carotin group." The discovery that carotin itself is a hydrocarbon led to the adoption of the name "carotene," as proposed by Arnaud (1886). The London Chemical Society favors the spelling "carrotene" for the hydrocarbon.

Zopf (1893a, 1895) proposed to distinguish between two groups of carotins, namely, eucarotins (true carotins) which were hydrocarbon in nature and carotinins, which contained oxygen as well, and formed compounds with the alkali and alkaline earth metals. It should be stated, however, that Zopf used the term carotin synonymously with lipochrome in most of his extensive studies of the pigments of the lower forms of plants and animals. His eucarotins, which were sometimes called yellow carotins, unquestionably contained representatives of our present group of xanthophylls whose chemical relation to carotin was not discovered until several years later. The carotinins of Zopf were red in color. The belief that they contained oxygen was based on the fact that they appeared to form alkali and alkali earth compounds. Obviously the carotinins are not related to the oxygen-containing xanthophylls, as known at the present time. None of the true carotinoids so far isolated in pure, crystalline state show acid properties like the so-called carotinins. The nature of the compounds which the latter are stated to form with sodium, calcium and barium remains to be determined, as well as their true relation to the carotinoids. The carotinins appear to be constituents of both plants and animals, as will appear from a fuller account of them given in Chapters III and V.

Tswett (1911a), to whose ingenuity we owe much of our knowledge regarding the physico-chemical properties of the chromolipoids, has proposed the term "carotinoide" for the various chromolipoids which are chemically and generically related to carotin. He would designate as carotins all those chromolipoids whose constitution and properties show themselves to be hydrocarbons, and as xanthophylls all those whose constitution and properties show themselves to be oxyhydrocarbons and which are chemically, as well as generically, related to carotin.

Tswett's terminology has been widely adopted. The author has also used it consistently in his own writings. The term carotinoid has the objection, however, that the -oid ending is derived from the Greek ειδυς, shape, so that strictly speaking the carotinoids are pigments which resemble carotin in form or structure only. As yet nothing definite is known regarding the structure of the carotinoids. The

word form cannot be restricted to crystalline form, inasmuch as the crystalline form of the carotinoids varies widely depending upon the solvent from which they separate. As will be pointed out later, however, the carotinoids must of necessity be closely related structurally. Their close chemical relations and the fact that they are invariably found together in chlorophyllous organs support this view.

Tswett's terminology has given promise of presenting a very simple solution of the difficulties of nomenclature in connection with the various red and yellow tinted pigments which conform to the properties of the so-called lipochromes so widely distributed in all forms of plant life. Unfortunately, however, Lubimenko (1914, 1915, 1916) has greatly complicated the system on very inadequate evidence by using the ending -oid for a group of pigments which he believes to correspond to each of the definitely known carotinoids. Thus, Lubimenko speaks not only of carotin, xanthophyll, lycopin, etc., but of carotinoids, xanthophylloids, lycopinoids, etc., as well. One cannot but express the opinion that our knowledge of the carotinoids in the sense used by Tswett, and followed in this monograph, is not sufficiently extensive to warrant a belief in the existence of numberless intermediate products. As a matter of fact, the chemistry of the specific individuals of Lubimenko's terminology, namely carotin, lycopin, xanthophyll and rhodoxanthin, argues against the existence of many plant chromolipoids of the nature of those mentioned. Certainly in view of the fact that there is every evidence to believe that all the xanthophylls bear the simple relation to carotin that is expressed in their respective formulae, $C_{40}H_{56}$ and $C_{40}H_{56}O_2$, it seems little short of preposterous to assume the existence of a group of "carotinoids" which are oxidation products of carotin and another group of "xanthophylloids" which are reduction products of xanthophyll.

Tswett's terminology, therefore, seems entirely adequate for our present knowledge of the chromolipoids of plant origin. If the chemical and physiological relation of the carotinoids to the yellow animal chromolipoids of the tissues and fluids of the higher mammals and man, and of the egg yolk and bodies of oviparous animals, is a criterion of similar relations throughout the entire realm of the animal kingdom, then Tswett's terminology is equally applicable to the yellow and red tinted chromolipoids so widely distributed in all forms of animal life. The probability of such a relationship is, in fact, the basis of the present monograph.

Non-carotinoid Plant Pigments

Carotinoids, however, are not the only yellow and orange colored pigments found in the plant and animal kingdoms, which fact must not be lost sight of in the examination of plant and animal products for the presence of carotinoid pigments.

Although all plant pigments have a hydrocarbon nucleus there are only a few yellow to red hydrocarbons known which are not carotinoids. They are the acenaphtylene of Behr and van Dorp (1873), Blumenthal (1874) and Graebe (1893), the di-biphenylenäthene of de la Harpe and van Dorp (1875) and Graebe (1892), the fulvenes of Thiele (1900a), the cinnamylidenindene of Thiele (1900b), and the rubicene of Pummerer (1912). Each of these is discussed more fully in Chapter IX, in connection with the probable constitution of carotin.

Among the naturally occurring yellow vegetable pigments which contain carbon, hydrogen and oxygen, but which have no relation to the xanthophyll group of carotinoids, two especially well defined groups are known, namely, the xanthones and the flavones. Five xanthones are known, (1) Cotoin, $C_{13}H_{12}O_4$, (2) Euxanthone, $C_{13}H_6(OH)_2O_2$, (3) Maclaurin, $C_{13}H_5(OH)_5O$, (4) Datiscetin, or di-methyl-tetraoxyxanthone, $C_{15}H_{12}O_6$, and (5) Gentisein, $C_{13}H_5(OH)_3O_2$. The structural constitution of each of these pigments is known. A much larger number of flavones are known, all of which are characterized by the common nucleus, β-phenyl-benzo-γ-pyrone. Many of the natural pigments occur as glucosides and are regarded as the chromogens from which anthocyanins are derived (Wheldale, 1916). Some of the more interesting members with a yellow color are (1) Luteolin, which is not to be confused with the carotinoid, Eteolin, but which is 1, 2, 3, 4-tetra-oxyflavon; and (2) Gossyptin, the yellow dye in the yellow flowers of the Indian cotton (*Gossypium herbaceum*), occurring there as a glucoside. No doubt the yellow color of cottonseed meal is due in part to Gossyptin, which can be extracted from cotton flowers with hot alcohol. The pure pigment exists as glistening yellow needles.

Besides the xanthones and flavones other yellow pigments are found in plant parts, among which may be mentioned chrysophanic acid, a methyl di-hydroxy anthracene whose solution in alcohol, ether, acetone, benzene, chloroform or petroleum ether will dye animal tissues a deep yellow color. Of special interest in this connection is the yellow pigment of the seeds of annatto (*Bixa orellana*), called bixin or

annatto, which is widely used for the artificial coloring of butter and cheese, and which has been commonly regarded as a lipochrome and was included by Thudichum among the luteins. The annatto pigment does, in fact, correspond in almost every particular with the class characteristics of the lipochromes. It is not entirely unattacked by alkalies, however, and furthermore is decomposable into a number of well known substances, such as *m*-xylene, *m*-ethyl toluene, and even palmitic acid. It reduces Fehling's solution even in the cold. Its constitution is unknown, as yet, but its elementary composition corresponds to the formula $C_{28}H_{34}O_5$, according to Etti (1878), or $C_{29}H_{34}O_5$, according to van Hasselt (1909). It is thus seen that bixin, while corresponding well to the lipochrome classification, is in no sense a carotinoid. Palmer and Kempster (1919c) have shown that the annatto pigment has no effect on the coloration of the tissues when fed to fowls.

Other vegetable coloring matters of a yellow color giving reactions in some cases similar to carotinoids, but of entirely different composition, are Crocin, and Crocitin, flavones which are found in the petals and pollen grains of the Indian crocus (*Crocus sativus*), which dissolve in concentrated sulphuric and nitric acids, with a deep blue color, which passes, however, into a brown shade. Another yellow vegetable dye showing a like reaction, although the after shade with the acid reagents is yellow, is the nyctanthin which Hill and Sikar (1907) described a few years ago. The empirical formula for this dye $C_{20}H_{27}O_4$, has an interesting resemblance to that of the carotinoids, at least when one doubles the above formula.

The yellow, orange, and red colors seen frequently among the fungi of the lichen and mushroom types appear to be due in many cases to pigments of a nature quite different from the carotinoids. Chrysophanic acid, mentioned above, sometimes occurs among these plants, as well as many other like coloring matters which have been named of Zopf (1889b, 1892b, 1893b) and which other workers have found occurring among the *Basidiomycetes*. In color and in some of their solubility properties these pigments resemble the carotinoids, and certain of them give a color reaction with concentrated sulphuric acid which is not unlike that regarded as characteristic of the lipochromes.

Non-carotinoid Animal Pigments

Several yellow pigments are present in animal tissues and fluids which are not to be mistaken for carotinoids. One of these, whose

constitution is unknown, but which is thought to be derived from the blood corpuscles, is hämatoidin, a yellow crystallizable pigment found in old blood exudates, in mummified embryos, and sometimes in the urine and other excreta. First described and named by Virchow (1847) and later by others, its origin as well as chemical properties and crystalline form have been recently studied anew by Neumann (1904, 1905). Holm (1867) thought the corpus luteum pigment was hämatoidin in his early study of this pigment which has since been shown to be carotin.

Another much more widespread yellow animal pigment with certain lipochrome properties is the bile pigment bilirubin. There is less danger of confusing it with carotinoids, however, save with respect to its color, inasmuch as it is a nitrogen containing substance which readily forms salts with the alkali and alkaline earth metals, and has many other properties at variance with those of the carotinoids.

Other non-carotinoid pigments exist in animal tissues, but which resemble the carotinoids in color and in solubility in fat solvents. Palmer and Kempster (1919a) have recently encountered such a pigment in the carotinoid-free egg yolks of hens raised from hatching on rations devoid of carotinoids, the eggs being produced likewise on xanthophyll-free rations. The small amount of pigment found in the yolks could be extracted by acetone, but hardly at all by ether, was almost entirely saponifiable and failed to respond to characteristic xanthophyll tests. The author finds that a similar pigment can be extracted from the carotinoid-free and apparently colorless "corpus luteum" of the sow, if a sufficient number of these organs are macerated and extracted with fat solvent. These cases are cited in order to point out the danger in assuming that all animal pigments of a yellow color are carotinoid in nature. Such a sweeping conclusion cannot be justified.

The same statement can also be made, although with less assurance, for certain red pigments which appear among the lower animals and birds. These pigments, as indicated, are red in the solid condition but their dilute solutions are usually yellow. They have been studied by certain of the older investigators, such as Kühne, Maly, Krukenberg, MacMunn and Zopf and others, and have received various names at the hands of these authors, such as rhodophan, vitellorubin, crustaceorubin, tetronerythrin, lina-carotin (from the *Lina* species of beetles in which they occur) and diaptomin. The pigments are strikingly similar in many respects to the carotinoids, but differ from them

in showing only one wide absorption band at F, and in forming, according to the statement of certain of their investigators, true compounds with sodium, calcium and barium. These points of divergence from the carotinoids should be examined in the light of our present knowledge of carotin and xanthophylls before it can be stated with assurance that these pigments are distinct substances. They all correspond completely to the class characteristics of the older terminology of lipochromes.

Summary

Red, orange and yellow pigments which have certain simple properties in common are found in almost all forms of plants and animals.

These pigments have been variously classified as luteins, lipochromes, lipoxanthins and chromolipoids.

These classifications have been based on general properties rather than on composition and are accordingly subject to both error and criticism.

As a general class term the name chromolipoid seems to conform more nearly to present conceptions of these pigments as well as to more common usage in connections with substances with fat-like properties.

Investigations regarding the composition of the chromolipoids show that a large majority of them are apparently chemically and generically related to carotin, a specific chromolipoid widely distributed in plant and animal tissues.

It seems reasonable to believe, therefore, that a great many chromolipoids can be classified more specifically as carotinoids, a name proposed for them by Tswett (1911a).

Two classes of carotinoids are recognized in Tswett's definition; carotins, whose constitution and properties show them to be hydrocarbons identical or isomeric with carotin; and xanthophylls, whose constitution and properties show them to be oxy-hydrocarbons and which are chemically, as well as generically, related to carotin.

Carotinoids are not the only yellow to red colored pigments occurring in plants and animals. Many of these non-carotinoids resemble the true carotinoids in one or more properties and some even in composition. The reader is referred to the text for the detailed discussion of the non-carotinoids and the properties which they have in common with the carotinoids as well as their distinguishing characteristics.

Chapter II

Carotinoids in the Phanerogams

There is no special reason, either physiological or genetical, for considering the carotinoids in the phanerogams and cryptogams separately, as is done in this and the subsequent chapter. In fact, there appears to be no logical reason for subdividing the plants into groups in connection with the distribution of carotinoids, inasmuch as the pigments appear to be widely distributed in all forms, both chlorophyllous and non-chlorophyllous, from bacteria to dicotyledons. The subdivision, then, is merely one of convenience.

The Pigments of the Carrot

The pigment of the carrot root (*Daucus carota*) was first described by Wachenroder (1826), nearly 100 years ago, and called carotin by him. This serves as the starting point of our knowledge of the properties, as well as the nomenclature of the carotinoids, and this pigment today represents our most typical chromolipoid. For this reason the carrot pigment will be considered first.

Wachenroder made an ether extract of the dried macerated roots, or the coagulum obtained on heating the carrot juice. The golden yellow salve-like residue left on evaporation of the solvent was shaken repeatedly with ammonia to separate admixed fatty material, dissolved again in ether and the ether allowed to evaporate slowly with addition of small amounts of alcohol. Ruby-red quadratic crystals, imbedded in fatty material, were obtained. Wachenroder described the crystals as tasteless and odorless, soluble in alcohol and ether, readily soluble in fats and ethereal oils, but insoluble in acetic acid and alkalis.

Vauquelin and Bouchardat (1830) are credited with the next study of the carrot pigment, but it was a number of years before Zeise (1847) isolated carotin from carrot roots in quantity sufficient for analysis. Zeise discovered the ready solubility of the pigment in carbon disulfide with its characteristic blood red color, as well as the fact that

alcohol when added to the concentrated carbon disulfide solution will throw down the carotin in crystalline form. The beautiful, glistening, copper colored crystals were described by Zeise, who also mentioned their insolubility in alcohol and their difficult solubility in ether and acetone. The crystals melted at 168° (+) C. Zeise made the first analysis of carotin and ascribed to it the formula C_5H_8. He was thus the first to show the hydrocarbon nature of the pigment, but due to the authority of the next investigator (Husemann (1861)), this fact was not proved until Arnaud (1886) made his careful analyses of the carrot pigment.

Husemann (1861) pressed the juice from finely grated carrots and then added weak sulphuric acid to the diluted juice, following Zeise's method, throwing down a coagulum which was partially dried and then extracted with hot 80 per cent methyl-alcohol. The residue was then dried completely and extracted with carbon disulfide. Carotin crystals were thrown out of the concentrated carbon disulfide solution by addition of absolute alcohol. Husemann purified the crystals merely by repeated washing on a filter with hot 80 per cent alcohol and finally with absolute alcohol. Husemann described the ruby-red color and velvety appearance of the carotin crystals, and their violet-like odor, which he found was especially noticeable on warming. He noticed the bleaching of the crystals in the air with the complete reversal of solubility, the bleached crystals being very difficultly soluble in carbon disulfide and benzine, but easily soluble in alcohol and ether. Husemann found that carotin was not precipitated by metallic salts but he observed the green color produced by adding ferric chloride to an alcoholic solution of the pigment. Palmer and Thrun (1916) have recently studied the reaction of ferric chloride on the carotinoids and have found it a most useful test for confirming the presence of these pigments in oils and fats and in various extracts of plant and animal tissues.

Husemann was the first to show the unsaturated nature of the carotin molecule, although he regarded the chlorine and iodine derivatives which he made as substitution products. Husemann's analyses led him to propose the formula $C_{18}H_{24}O$ for carotin and his figures were accepted over those of Zeise.

Arnaud (1886) was the next investigator of the carrot chromolipoid. He isolated the pigment from 600 kilograms of carrots by pressing the juice from the grated roots, adding lead acetate to the juice, drying the precipitate in vacuum and extracting it with carbon disulfide. The

dried press cake was extracted similarly and the carbon disulfide distilled off of the combined extracts. The residue was washed with cold petroleum ether and the pigment purified by crystallization from carbon disulfide with absolute alcohol and then allowing it to crystallize spontaneously from cold petroleum ether. About 3 grams of crystals per 100 kilograms of carrots were obtained in this way.

Arnaud found the bleaching of carotin noticed by Husemann to be an oxidation, analyses which he made of the bleached product showing an addition of 21 per cent oxygen. The rapid bleaching of carotin solutions was also noticed; and Arnaud pointed out the influence of this fact on the securing of pure preparations for analysis. Arnaud's analyses of freshly prepared crystals showed an average composition of 88.67 per cent carbon and 10.69 per cent hydrogen, definitely proving the correctness of Zeise's assertion regarding the hydrocarbon nature of the pigment. This investigator was also the first to prepare the crystalline iodine derivative of carotin by adding iodine crystals a little at a time to a solution of carotin in anhydrous petroleum ether, maintaining the while an excess of carotin in the solution. It was the elementary composition of this product, considered together with the composition of the pure carotin, that led Arnaud to ascribe to carotin the formula $C_{26}H_{38}$, and to the iodine derivative the formula $C_{26}H_{38}I_2$.

Kohl (1902b) has given us one of the most detailed descriptions of the chemical and physical properties of carotin. His analyses of the crystalline pigment, however, gave unsatisfactory results, as did also his molecular weight determinations, using the cryoscopic method. He therefore accepted Arnaud's formula as representing the correct composition of carotin. Certain of Kohl's detailed descriptions of carotin will be summarized in Chapter IX, where the physical and chemical properties of the carotinoids are considered.

Willstätter and Mieg (1907) definitely settled the composition of the carrot carotin at the time they proved its identity with the carotin of the chloroplastid. Their data show a mean ratio of C:H of 1:1.406 for the carrot carotin, for which the simplest formula is C_5H_7. Molecular weight determinations in $CHCl_3$ and CS_2, using the ebulloscopic method, show an average of 536, which corresponds exactly with the formula $(C_5H_7)_8$, or $C_{40}H_{56}$, which thus appears to be the correct formula for carotin.

Escher (1909) and Willstätter and Escher (1910) have confirmed these results completely. Escher furthermore attempted to ascertain

the structure of carotin using 150 grams of the pigment isolated from carrot meal by extraction with petroleum ether. His efforts led only to the production of amorphous products, all of high molecular weight. The constitution of the pigment thus remains to be determined.

Euler and Nordenson (1908) also isolated carotin from carrots in quantity sufficient for analysis. Their results confirm the Willstätter formula. The purified crystals from 25 kilos of fresh carrots were found to contain xanthophyll, which was identified by the color of the crystals and their solubility properties. Palmer and Eckles (1914g) have also shown the presence of xanthophyll carotinoids in the carrot root by the Tswett (1906c) chromatographic method of analysis, but van Wisselingh (1915), using microchemical crystallization methods, did not observe any xanthophyll crystals.

It appears that anthocyanins, also, may accompany carotinoids in the carrot root. Wittmack (1904) has described a red variety of carrots (*Daucus carota,* var. *Boissieri Schweinfurth*) which contains both carotinoids and anthocyanin.

Many other investigators have isolated carotin crystals from carrots without, however, submitting them to chemical examination. According to Schimper (1885) and Courchet (1888) carotin exists in the carrot tissue in crystalline form. Van Wisselingh (1915), however, has shown that the little tubules which Schimper and Courchet observed are not true crystalline forms. The author, also, has never observed any but granular deposits of carotin in sections of the fresh carrot tissue.

Carotinoids in Other Roots

Very few other roots have been examined for carotinoids although several which are widely used as food are characterized by their yellow color, e.g., the yellow parsnip root (*Pastinaca sativa*), and the sweet potato (*Ipomoea batatas*), especially the highly colored varieties grown in the southern part of the United States, popularly called Yams. The pigments of these roots should be examined.

Formanek (1900) has studied the pigment of the red beet (*Beta vulgaris*), and believes that the red pigment changes into a yellow one under certain conditions. The absorption bands of the latter are identical in position with those of carotin. Formanek's red pigment showed only one absorption band in the yellow part of the spectrum and is undoubtedly an anthocyanin. Its apparent transformation into carotin cannot at present be explained.

Lubimenko (1914a) has examined the pigment of the yellow turnip root (*Brassica Rapa* L.) and finds that it contains a yellow pigment soluble in 95 per cent alcohol, but which he was not able to crystallize, and also a pigment which closely resembles lycopin, the red pigment of the tomato. Spectroscopically the pigment appears to be identical with lycopin but because of a difference in the relative intensity of the bands as compared with lycopin, and a greater ease of solubility in alcohol and concentrated acetic acid, Lubimenko preferred to call the pigment a lycopinoid, a term which the author regards as very unfortunate in view of the more generally accepted use of the terminology -oid as applied to the carotins and xanthophylls.

It seems possible that the pigment of the related variety of turnips, namely, rutabaga (*Brassica campestris* L.) is of the same character. The question of this type of carotinoid in roots deserves confirmation and further study.

Carotinoids in the Chloroplastids

The tissues of all chlorophyllous plants are characterized by certain specialized bodies, probably protein in nature, of microscopic size, called plastids. In early stages of the plant's development and often in the subterranean parts of the plant after maturity the plastids are colorless. They are then called leucoplastids. More commonly they develop green pigments, chlorophylls, when the plastids are called chloroplastids, or chlorophyll granules. The chlorophylls in the chloroplastids are always accompanied by carotinoids of both types, namely, carotin and xanthophylls.

Investigations regarding these yellow chromolipoids in the chloroplastids apparently did not begin until the observation of Frémy (1860) that a yellow pigment can be obtained from green leaves by allowing strong HCl and ether to act upon the residue from the alcoholic extract, or by similar treatment of the precipitate thrown down from the alcoholic leaf extract by $Al(OH)_3$. In this procedure the ether took on a yellow color, the pigment of which Frémy called phylloxanthine, leaving a blue pigment, which he called phyllocyanine, in the aqueous acid layer. Frémy believed that his phylloxanthin pre-existed in the leaves.

It is now quite certain that Frémy's phylloxanthine was a mixture of some of the natural carotinoids of the leaf with an acid decomposition product of chlorophyll, a view which was expressed first by Stokes (1864). The name phylloxanthin is, in fact, at present re-

served for a product of rather indefinite composition which results from the action of acid on chlorophyll b (Tswett, 1907, 1908b).

In a later study of methods of isolation of phylloxanthine, however, Frémy (1865) undoubtedly obtained much more valid proof of the existence of yellow pigments associated with chlorophyll although he regarded the pigment which he isolated as the same yellow phylloxanthine isolated by the ether-HCl method. He found that a careful addition of $Mg(OH)_2$, or $Al(OH)_3$ to alcoholic chlorophyll solutions carried down the green pigment only, leaving the yellow pigment in solution. $Ca(OH)_2$ and $Ba(OH)_2$ gave similar results, but the best procedure with the last named reagent was to add an excess, which threw down all the pigments, from which the phylloxanthine (carotinoids) could be extracted with alcohol. Especially interesting was Frémy's observation that when his chlorophyll was saponified with strong bases, alcohol took up the yellow "phylloxanthin" from the residue, and yellow plate-formed or reddish colored prismatic crystals, soluble in alcohol and ether, could be obtained from this solution. The red crystals were described as being very much like crystals of potassium dichromate, and having a strong coloring power. It would appear as though Frémy succeeded in obtaining for the first time crystals of carotin, and possibly xanthophyll also, from green plants.

Fremy's observations precipitated a lively interest in the subject of yellow pigments in the chloroplastids which resulted in a number of investigations during the succeeding years, some quite independent of the others. These investigations seem to fall quite naturally into several groups. The first of these was a series of studies confirming the presence of yellow substances accompanying chlorophyll through the development of suitable methods for their separation.

Separation of Yellow Pigments from Chlorophyll

Stokes (1864a) is to be credited with first discovering a method for separating the actual yellow pigments accompanying chlorophyll and for recognizing the existence of distinct green and yellow constituents in the plastids. This investigator states, "I find the chlorophyll of land-plants to be a mixture of four substances, two green and two yellow, all possessing distinctive properties"; and later referring to phylloxanthine he states, "When prepared by removing the green bodies by $Al(OH)_3$ and a little water, it (phylloxanthin) is mainly one of the yellow bodies, but when prepared by HCl and ether, it is a

mixture of the same yellow body (partly, it may be, decomposed) with the product of decomposition by acids of the second green body." Stokes never published his method of separation in detail but he gives a hint of its character in a paper in another publication (1864b), in which he states in a discussion of the advantages of a partition between solvents for the separation of various substances, "Bisulphide of carbon in conjunction with alcohol enabled the lecturer to disentangle the colored substances which are mixed together in the green coloring matter of leaves."

Stokes was not the only one of the earlier investigators to express the belief that Frémy's pigments were decomposition products. Filhol (1865) also reached this conclusion, as did Askenasy (1867). Filhol (1868) a little later noticed that it is possible to remove the green constituent of crude alcoholic chlorophyll solutions by treating them with animal charcoal insufficient to completely decolorize the solution. A yellow colored solution remained on filtering off the bone-black, the color of which Filhol believed to be due to a pre-existing pigment or pigments associated with the green one. C. A. Schunck (1901), many years later, employed this method of obtaining his xanthophyll group of pigments free from chlorophyll. Schunck's "xanthophylls" included carotin also, so that Filhol's observation was in reality of much more importance than he realized.

Timiriazeff (1871), studying Frémy's phylloxanthin, also found that alcohol alone would extract the yellow pigment from the barium compound thrown down from alcoholic leaf extracts by an excess of $Ba(OH)_2$. He preferred to call the yellow pigment xanthophyll, the name previously employed by Berzelius (1837a)—from χανδός, yellow and φύλλον, leaf—for the yellow pigment which he extracted from the yellow autumn leaves of the pear tree (*Pyrus communis*). Sorby (1871b) employed the same term for a group of yellow and orange pigments which, with chlorophyll, he believed caused the green color of leaves, and were represented as types by pigments which could be extracted from carrots by CS_2.

Notwithstanding the previous observations of Frémy, Stokes, Filhol, Timiriazeff and Sorby, credit is given in most quarters to Gregor Kraus (1872a) for making the first actual separation of the pigments of leaf extracts from one another. Kraus' method is frequently referred to as an "ausschüttlungs" method, for he shook the green alcoholic leaf extracts with benzene and observed that the benzene had extracted the green pigment leaving the alcohol layer a beautiful

yellow. Kraus named the green pigment cyanophyll and the yellow pigment xanthophyll. From our present knowledge of the relative solubility properties of the carotinoids which have been built very largely upon Kraus' observations, it is obvious that his xanthophyll pigment was composed almost entirely of the xanthophyll carotinoids, although traces of carotin may have been present also. Kraus found that the absorption bands of his xanthophyll fraction in the blue part of the spectrum corresponded with Bands V and VI of the leaf extract. The residue from Kraus' xanthophyll fraction gave a deep blue coloration with concentrated sulphuric acid, and the xanthophyll solution itself bleached very quickly in the sunlight.

Several studies of the leaf pigment, using the Kraus procedure, soon followed. Konrad (1872) observed that the separation of xanthophyll from chlorophyll by benzene was effective only when an alcohol of about 70 per cent (by volume) strength was employed. Treub (1874) substantiated the necessity of using weaker alcohol for the benzene separation, and found that CS_2 was effective when the chlorophyll extract was in strong alcohol. Cempert (1872) found that linseed oil could be used in place of benzene and Wiesner (1874a, b) found that a number of vegetable oils (linseed, walnut, poppyseed, olive) and ethereal oils (turpentine oil, rosemary oil, oil of wintergreen) and also carbon disulfide could be employed. Wiesner used 85 per cent (by volume) alcohol and benzene, boiling at 92-94° C., for the regular Kraus separation. Equally good results were obtained with toluene and xylene, or mixtures of these with benzene. Wiesner even succeeded in shaking the green chlorophyll out of alcoholic leaf extracts with dilute egg albumin. Especially important was his observation that dilute ammonia or caustic alkali solutions acting on the residues from alcoholic leaf extracts would take up most of the yellow color leaving behind the chlorophyll, mixed with a little xanthophyll. This observation, pointing to the resistance of the yellow chromolipoids to alkalis and the attacking of chlorophyll by the same reagents, was later developed by Hansen (1884a) and is still widely used for the separation of carotinoids from chlorophyll. Hansen boiled young wheat plants in water for one-half hour, dried the product, extracted with cold 96 per cent alcohol in the dark, concentrated the extract to one-eighth volume, saponified with NaOH, diluted the soap solution with water, and salted out the soap with NaCl. The green soap thus obtained was extracted with petroleum ether, which gave a yellow extract which Hansen called "chlorophyll gelb," the residue being

called "chlorophyll grün." Carl Kraus (1875) observed the same facts when he found that benzene extracts a yellow color from alcoholic leaf extracts made strongly alkaline with KOH. He called the yellow pigment xanthin and regarded it as a decomposition product of G. Kraus' xanthophyll.

Possibly following the hint given by Stokes (1864b), Sorby (1873) developed a separation method for the yellow and green constituents of a number of types of plants using alcohol and carbon disulfide. Sorby named five members of a "xanthophyll" group of yellow pigments as well as two chlorophylls, but pure pigments could not have been obtained in most cases, since the methods which he employed will not give a true separation of the various carotinoids of the chloroplastids. Of the various pigments named by Sorby the "yellow chlorophyll" is obviously a xanthophyll mixed with some chlorophyll, and the "orange xanthophyll" is for the most part carotin. Sorby's "xanthophyll" and "yellow xanthophyll" are the only true xanthophylls, the former being in all probability a mixture of what are now called α and α′ xanthophyll, while the "yellow xanthophyll" apparently consisted almost wholly of our present β xanthophyll, which is characterized, as Sorby found for it, by the development of a blue color when its alcoholic solution is treated with HCl.

Crystalline Carotinoids from Chloroplastids

The second group of studies relating to the yellow pigment in the chloroplastids deals with isolation of crystals of carotinoids, and terminated with the isolation and analysis of one of the pigments and the discovery of its identity with the carotin of carrots. The various studies were, for the most part, independent of each other and accordingly resulted in the proposal of several different names for the chloroplastid chromolipoids.

Frémy's (1865) observations regarding crystalline chromolipoids have already been mentioned. He apparently regarded the crystals as related to his phylloxanthin, as no special name was proposed by him for the crystalline pigment. Hartsen (1873a), however, several years later, observed golden-red crystals in the deposit from the spontaneous evaporation of an alcoholic extract from green leaves. He called the pigment chrysophyll, a name previously applied by Sorby (1871b) to a group of water-soluble pigments from autumn foliage, and later (1875) described a method for purifying the crystals by

washing away the fat and chlorophyll with petroleum ether, taking up the pigment in alcohol and recrystallizing. Hartsen regarded his chrysophyll as probably identical with xanthophyll (G. Kraus) and as existing together with chlorophyll in the leaf. According to Willstätter and Mieg (1907) Hartsen's chrysophyll was probably a xanthophyll in the sense that this name is used at the present time, but most writers have regarded it as identical with carotin. Bougarel (1877), a little later, isolated red crystals from the alcoholic extract of peach and sycamore-tree leaves. He described the insolubility of the red, green reflecting crystals in alcohol and ether, and their solubility in chloroform, benzene and carbon disulfide, as well as the rose color of the solution in the last named solvent. Notwithstanding his familiarity with Hartsen's chrysophyll, which he mentions, Bougarel regarded his pigment crystals as a new substance and unfortunately proposed the name erythrophyll for it, which had already been given by Berzelius (1837b) many years before for the red, alcohol soluble pigment which he isolated from red cherries (*Prunus cerasus*), black Johannis berries (*Ribus nigrum*) and the red autumn leaves of various plants, and which was also used by Sorby (1871, 1873) for a group of water-soluble pigments. The erythrophyll of Bougarel is unquestionably to be regarded as carotin.

Dippel (1878) made a careful study of the absorption spectra of G. Kraus' xanthophyll and cyanophyll and the products of the action of KOH and acid on the pigments prepared according to the Kraus method. He found that yellow pigments could be prepared in each case but that the absorption spectrum of the yellow pigment from the acid treatment of cyanophyll was entirely different from the spectra of the yellow pigment from the alkali treatment of both xanthophyll and cyanophyll. Dippel proposed the name xanthin (compare C. Kraus (1875)) for the yellow pigment obtained from Kraus' xanthophyll and cyanophyll on treatment with alkali and extracting with alcohol, and regarded it as the true yellow constituent of chlorophyll. The absorption bands of Dippel's xanthin obtained by alkali treatment of Kraus' benzene-cyanophyll layer lay at 490-456μμ and 455-435μμ, while the bands of Kraus' xanthophyll, as measured by Dippel, lay at 483-460μμ and 446-433μμ. These measurements correspond almost exactly with those of carotin and xanthophyll, respectively, as known at the present time. Dippel's xanthin is to be regarded, therefore, as composed of carotin for the most part.

Borodin (1883) made one of the most striking contributions to our

knowledge of crystalline carotinoids accompanying chlorophyll in the chloroplastids. Two groups of pigments were described by him, one characterized by their slight solubility in alcohol and great solubility in benzine, corresponding with one of the properties of carotin, the other group being characterized by their ease of solubility in alcohol and their slight solubility in benzine, which corresponds with one of the most distinguishing properties of the xanthophylls. No names were proposed by Borodin for his crystalline pigments but he described in detail the crystal forms and certain properties of two carotins and two xanthophylls. One of the carotins formed orange-red rhombic crystals (he obtained these from the alcoholic extract of *Spirogyra*) and the other bright yellow needles with a strong violet or rose-red nuance. Of the two xanthophylls one formed straw yellow, ribbon-like scales or dark brown, crooked, branching rods, and the other golden-yellow "navikeln," an English synonym for which the author has not been able to find. The latter were observed especially clearly by Borodin in extracts from parsley (*Petroselinum sativum*). Borodin regarded the red and violet tinted, benzine-soluble group as widely distributed in all chlorophyllous plants, the red forms being identical with Bougarel's erythrophyll. The alcohol soluble forms were not regarded by Borodin as being so widely distributed, especially the pigment forming the golden-yellow "navikeln." With the exception of the red rhombic-formed crystals in the benzine-soluble group Borodin's crystal forms do not correspond with the carotins and xanthophylls which have since been isolated in pure form by various investigators so it is not known whether they represent forms which were modified by the solvents employed or isomeric carotin and xanthophyll carotinoids not yet isolated in quantity. The latter possibility is not to be disregarded in view of the various yellow chromolipoids which are revealed in chloroplastids using Tswett's (1906c) chromotographic analytical procedure.

Guignet (1885) observed orange crystalline material from extracts obtained by a method similar to that used by Dippel for xanthin. Alcoholic leaf extract in strong alcohol was agitated with one-tenth its volume of petroleum ether (Sachsse (1877) introduced the use of petroleum ether instead of benzene in the Kraus separation) and the green petroleum ether extract agitated with a solution of NaOH in 95 per cent alcohol, leaving a yellow solution from which the crystalline material was obtained. The pigment was no doubt carotin although no name was proposed for it by Guignet.

It remained for Arnaud (1885), however, to first recognize and prove the identity of the orange-red crystals apparently first observed by Frémy (1865) and later called chrysophyll, erythrophyll, xanthin, etc., with the carotin from carrots, which had been known and studied for 60 years. Arnaud (1885) first observed the identity in form and properties of carotin which he isolated from carrots and the red rhombic crystals which he isolated from spinach leaves (*Spinachia oleracea* and *glabra*), mulberry leaves (*Morus alba*), the leaves of peach (*Persica vulgaris*) and sycamore (*Acer pseudoplatanus*) trees, and the leaves of the English ivy vine (*Hedera helix*), as well as from pumpkins (*Cucurbita pepo*). In a succeeding paper Arnaud (1886) proved this identity by his analyses of the crystals obtained from carrots, to which reference has already been made, the results leading to the proposal of the formula $C_{26}H_{38}$ for the pigment. Arnaud did not make any analyses of the apparently identical crystals which he obtained from leaves, so that strictly speaking the final proof of the identity of the crystals was not furnished until Willstätter and Mieg performed their comparative analysis many years later (1907). However, following Arnaud, investigators with few exceptions adopted his terminology and called the red crystalline pigment carotin which could be isolated so generally from chlorophyll forming plants, as well as many fruits and seeds, and from cryptogamic forms. Immendorff (1889), in fact, soon after Arnaud's work, isolated carotin from barley and rye leaves and submitted the crystalline pigment to analysis. His data corresponded best with Zeise's older formula, C_5H_8, but he preferred to accept the Arnaud formula because it appeared to be substantiated by Arnaud's analysis of the iodine derivative of carotin.

Willstätter and Mieg, however, starting with 100 kilos of dried nettle (*Urtica*) leaves, isolated carotin in sufficient quantity to establish for it the correct formula, $C_{40}H_{56}$. Their analyses gave the average composition of 89.29 per cent carbon and 10.53 per cent hydrogen as compared with the theoretical values 89.48 and 10.52 per cent carbon and hydrogen, respectively.

Plurality of Yellow Pigments in the Chloroplastids

The next group of investigations dealing with the yellow pigments of the chloroplastids had to do with the question whether more than one yellow pigment is a constant accompaniment of the chlorophyll. This question brings us up to the present time for notwithstanding the

fact that two crystalline carotinoids in the xanthophyll group have now been isolated from plant forms and their composition determined, the isolation of other known members of this group of pigments still remains to be carried out and their composition and relation to the known members determined.

Stokes (1864a) is to be credited with the first suggestion of the presence of more than one yellow pigment in chloroplastids, but in spite of the various yellow pigments isolated by Sorby (1873), using Stokes' carbon disulfide procedure, this method could not have led to a true isolation of the various members of the carotinoid pigments which are recognized today. The observation of Dippel (1878) that besides Kraus' xanthophyll a yellow pigment accompanied the cyanophyll in the petroleum ether layer, could have led to the discovery of the actual existence of two groups of carotinoids. Borodin (1883), however, first demonstrated the existence of more than one yellow chromolipoid when he obtained various forms of crystals from green plants. As already pointed out, these crystals naturally fell into two groups according to their solubility properties, one group, to which Borodin recognized the erythrophyll (carotin) of Bougarel belonged, being very soluble in benzine (petroleum ether) and difficultly soluble in alcohol, and the other group being easily soluble in alcohol but dissolving with difficulty in benzine. These observations of Borodin's are the basis of the classification of the carotinoids which prevail at the present time, considerably extended and supported, of course, by other chemical and physical properties; they also furnish the basis for the separation of the carotinoids into the two groups now recognized, namely, the carotin and xanthophyll groups. This system of classification is the only logical one, as has been pointed out by Tswett and proven by the chemical analyses of members of each group by Willstätter and his co-workers. Before reviewing the history and evidence in favor of this classification and the proof of the existence of individuals in the group, it should be stated that the plurality of yellow chromolipoids in chloroplastids has been recognized by other investigators who have proposed other systems of classification. These latter studies will be reviewed first.

Tschirch found proof of the existence of more than one yellow chromolipoid in his well-known series of spectroscopic studies of chlorophyll. In his first papers Tschirch (1884, 1885, 1887) considered the yellow constituents of the chloroplastid to be erythrophyll (adopting Bougarel's terminology) and a group of five xanthophylls,

which he called α, β, γ, δ and ϵ xanthophyll, respectively, although he concluded from the fact that each pigment showed two absorption bands showing no significant spectro-analytical differences that they were probably identical substances. Kohl (1902c) later considered all these xanthophylls to be carotin, but a comparison of the measurements of the spectrum bands of γ and δ xanthophyll as given by Tschirch (1885) would indicate that the former may have been due to carotin, but that the latter was undoubtedly one of the xanthophylls as now recognized.

In his next paper Tschirch (1896) regarded all the yellow chromolipoids as xanthophylls and distinguished between two, one of which was obtained in metallic glistening crystals, which he called xanthocarotin. This pigment showed three beautiful absorption bands, the measurements of which correspond with those now recognized for xanthophyll. The other xanthophyll could not be obtained in crystalline form and its solutions were characterized by showing no absorption bands, only end absorption of the violet and ultra-violet. Tschirch used fresh grass as the source of his material for this study.

In his most recent paper Tschirch (1904) turned his attention to a comparison of his xanthocarotin and xanthophyll with the carotin from carrots. Spectroscopic absorption properties only were considered. There has always been a question in the author's mind as to which group of carotinoids Tschirch's xanthocarotin belongs. Tschirch himself considered that it might be identical with the carotin from carrots, inasmuch as the absorption spectra of the crystalline pigment which he isolated from carrots and that of his xanthocarotin from grass were identical. Kohl (1902d) believed that Tschirch's xanthocarotin was carotin contaminated with phytosterin, and Tswett (1911a) apparently also regarded the pigment as a carotin although he recognized the absorption spectra of Tschirch's carrot carotin did not correspond exactly with the measurements given by other investigators. Willstätter and Mieg (1907) also regard the xanthocarotin as carotin, but with these views the author is not in agreement on the following grounds. The author believes that Tschirch's carotin crystals from carrots were xanthophyll, not carotin, for he obtained them merely by spontaneous evaporation of an ether extract of sugar-free carrots, which would be more likely to yield xanthophyll crystals than carotin. Moreover, the crystals had the reddish yellow color and steel blue reflection described by Willstätter and Mieg for crystals of xanthophyll. In addition the absorption spectra of these crystals correspond

exactly with those of xanthophyll, not carotin, as the following table shows:

Carotin in alcohol (Willstätter and Stoll (1913a))	*Xanthophyll in alcohol* (Willstätter and Mieg (1907))
I—492–476 μμ	I—488–471 μμ
II—459–445 "	II—454–440 "
III—430–419 "	III—425–420 "
Carrot carotin in alcohol (Tschirch)	*Xanthocarotin in alcohol* (Tschirch)
I—487–470 μμ	I—485–468 μμ
II—457–439 "	II—455–438 "
III—429–417 "	III—430–418 "

Much discussion has also resulted from the statement made by Tschirch in the paper under consideration that he was able to observe the transformation of xanthocarotin into xanthophyll. As a matter of fact Tschirch observed merely that certain impure xanthocarotin solutions lost their absorption bands without losing their color appreciably. In view of the fact that the so-called xanthophyll of Tschirch showed no absorption bands but merely end absorption, he concluded that xanthocarotin readily changes over into xanthophyll, a most sweeping conclusion from such indefinite evidence. The author has observed many times that impure solutions of carotinoids lose their spectroscopic absorption bands in the earliest stages of decomposition with little or no loss in color of the solutions.

A somewhat different system of yellow chromolipoids was proposed by Schunck (1899, 1901, 1903) in his series of papers. He depended largely upon the spectroscopic absorption properties of the pigments for their differentiation, as did Tschirch, and in his later studies upon the action of certain chemical agents upon the absorption bands. It may be stated of Schunck's work, faulty as it was in certain respects, particularly in his adoption of Sorby's method for separating the various yellow coloring matters by carbon disulfide, that he has given us some of the most beautiful spectro-photographs of the carotinoids that exist in the literature. Schunck accepted from the outset that more than one yellow chromolipoid was present in the chloroplastids. Inasmuch, however, as he modified his views somewhat regarding the number and nomenclature of these pigments during the course of his studies his final views only will be discussed.

Schunck proposed to call all the yellow pigments accompanying chlorophyll xanthophylls, the chief member of the group being chrysophyll, thus adopting Hartsen's terminology for carotin in spite of the fact that Schunck not only referred to Arnaud's work but confirmed

it from a spectroscopic standpoint. Besides chrysophyll, the only member of the "xanthophylls" which he was able to obtain in crystalline form, Schunck separated two other xanthophylls from green leaves by shaking the alcoholic chlorophyll-free [1] solution with successive equal portions of carbon disulfide, each volume of carbon disulfide being equal to about one-half the volume of the crude solution experimented upon. This was continued until no more color was extracted, three or four extractions being sufficient as a rule to accomplish this result.

With the exception of the first carbon disulfide extract, which contained the crystallizable chrysophyll as well as one of the xanthophylls, Schunck erroneously believed that the various carbon disulfide fractions represented more or less pure solutions of individual xanthophylls with varying degrees of relative solubility in alcohol and carbon disulfide.

The various carbon disulfide fractions were now allowed to evaporate spontaneously, the residue was taken up again in alcohol and the spectroscopic absorption bands photographed. The effect on these bands of adding concentrated HCl, HNO_3, H_2SO_4, H_2O_2 and nascent hydrogen was studied, as well as the effect of these reagents on the color of the alcoholic solution. Certain marked differences were observed with the various fractions.

The first fraction from green leaves contained, besides chrysophyll, a pigment which Schunck called L. xanthophyll, whose spectroscopic absorption bands differed from those of chrysophyll by being shifted only slightly towards the ultra-violet and whose solution, like chrysophyll, changed to a green tint before fading on addition of HCl or HNO_3, the absorption bands disappearing.

The subsequent carbon disulfide extracts contained a second xanthophyll, called B. xanthophyll, which differed from the first in two respects, (1) the absorption bands (Schunck observed three distinct bands for all his "xanthophylls") were shifted slightly more towards the ultra-violet, (2) the effect of acids on the alcoholic solution was to produce a brilliant green color which gradually changed to a beautiful peacock blue, then purple, and gradually bleached entirely. Especially striking was the observation that the addition of ammonia

[1] This was obtained by one of two methods, either by adsorbing the chlorophyll on animal charcoal, which does not remove the "xanthophylls," according to Schunck, or by saponifying the alcoholic leaf extract and extracting the soap with ether, the latter taking out the yellow pigments. After evaporation of the ether the pigments were taken up in alcohol for the "xanthophyll" separations.

to the blue solution restored the original yellow color of the solution, although less intense, the blue color reappearing on acidifying again. Sorby (1873) mentioned this reaction for his "yellow xanthophyll." The author[2] has observed that the change from yellow to blue and *vice versa* can be repeated apparently indefinitely with one of the xanthophylls obtained from plants by Tswett's chromotographic method.

In his last paper Schunck found evidence of the existence in flowers of still another xanthophyll, called Y. xanthophyll, with properties similar to B. xanthophyll, except that it was much less readily extracted from alcohol by carbon disulfide and was accordingly found in the alcohol after the carbon disulfide extractions. Schunck found no evidence of the existence of Y. xanthophyll in his leaf extracts.

Kohl (1902e) attempted to harmonize the views of Tschirch and Schunck as well as his own belief that carotin is the principal pigment in the chloroplastids. He recognized the difference between carotin and the xanthophyll proper of Schunck, but apparently did not recognize the existence of several of these xanthophylls, as proposed by Schunck. Kohl recognized also the existence of the xanthophyll of Tschirch, which showed no absorption bands, and believed, like Schunck, who proposed no name for the pigment, that it could be extracted from the chloroplastids by hot water, as well as by alcohol. Kohl, therefore, proposed to call Schunck's xanthophyll α xanthophyll and the xanthophyll of Tschirch β xanthophyll, and expressed the belief that carotin and these two xanthophylls comprised the yellow pigments in the chloroplastids.

We will now return to a consideration of the investigations leading up to the classification of the carotinoids which prevails at the present time. Following Borodin, Monteverde (1893) found that the yellow pigments accompanying chlorophyll can be divided into two groups according to their relative solubility in alcohol and petroleum ether, and he was the first to show that this fact offers a very simple means of separating the pigments from each other. Using the procedure of Frémy and Timiriazeff, Monteverde precipitated the chlorophyll from an alcoholic leaf extract with an excess of $Ba(OH)_2$, which carries down with it the carotinoids also, and extracted the yellow pigments from the precipitate with alcohol. Petroleum ether and a few drops of water were added to this yellow solution, and the mixture shaken. The liquids soon separated into two layers, each con-

[2] Unpublished observation.

taining a yellow pigment with distinguishing characteristics. Mo
verde found the pigment in the upper petroleum ether layer to
spectroscopically as well as in other respects identical with car
and accordingly called it carotin. The pigment remaining in
alcohol layer, on the other hand, was found to be different in m
respects and was called xanthophyll, following Gregor Kraus' ter
nology. Monteverde regarded it as not unlikely that this "xant
phyll" itself consisted of two yellow pigments. In order to sepa
completely the carotin and xanthophyll the petroleum ether
alcohol layers after separation were shaken with fresh quantities
alcohol and petroleum ether, respectively. On spontaneous evapo
tion of the alcoholic xanthophyll solutions Monteverde obtained cr
tals which corresponded exactly in form with the "strohgelben Kr
tallen" described by Borodin. There is some doubt, however, whet
the pale yellow crystals observed by Monteverde, and the similar o
observed by Borodin, were actually xanthophyll. Reinke (1885) s
eral years previously obtained yellow platelets on evaporation of al
holic solutions of the yellow chloroplastid pigments and found th
to be merely phytosterol or a mixture of sterols colored with pigme
It is likely that Monteverde was misled by the same phenomenon,
the great solubility of xanthophyll in alcohol undoubtedly preve
the formation of crystals when one is dealing with the very sm
quantities of pigment present in Monteverde's solutions. Montever
however, described very clearly the difference between the absorpt
spectra of carotin and xanthophyll, as did Schunck, some years lat
between chrysophyll (carotin) and the L. B. and Y. xanthophylls wh
he separated. Monteverde also described the green coloration, chan
ing to a blue on addition of concentrated HCl to the alcoholic xa
thophyll solution, a reaction which also characterized the B. and
xanthophylls of Schunck, as mentioned in an earlier paragraph.

Tswett was very quick to recognize the importance of Monteverd
work and the significance of the Kraus method of separation in in
cating the existence of alcohol-soluble xanthophylls in contrast w
benzine-soluble carotin. This investigator's keen appreciation of
significant properties of carotin and xanthophylls is what makes p
sible today the extension of our knowledge of the distribution
these pigments in all forms of plant and animal matter. Tswet
important observations are accessible to us in a series of pap
(1906a, b, c, 1911a) from 1906 to 1911. The last paper is more
the nature of a summary but by reason of its clear-cut statements

may well serve today as our best laboratory outline for working with the class of pigments with which this monograph deals. It was in this paper that Tswett proposed the nomenclature for the carotinoids which has been adopted in this monograph.

Tswett's most important contribution to the subject, from an investigational standpoint, was on certain physico-chemical properties of the pigments. He showed (1906b) that the various colored constituents of the chloroplastids, when carefully obtained in certain solvents by methods which avoid the action of plant acids, exhibit very characteristic adsorption coefficients towards finely divided materials, such as $CaCO_3$, inulin and sucrose, as well as many other inert materials which are insoluble in the solvent employed and which can be obtained in a finely divided state. This exceedingly interesting phenomenon is no doubt due to the fact that the various green and yellow chromolipoid constituents of the chloroplastids exist in organic solvents in colloidal aggregates of various sizes, the larger colloidal particles being the more strongly adsorbed, and some, like carotin, which is not adsorbed at all, existing in true solution. Tswett found petroleum ether, the carotin solvent, to serve best for the study of these properties, although carbon disulfide was also very useful because of the brilliant color which all the chloroplastid pigments show in this solvent, and also because the xanthophylls are especially well differentiated in this solvent. This latter fact is no doubt closely related to Schunck's (1903) observations regarding the relative solubility of xanthophylls in carbon disulfide by which he believed he was able to separate them from one another by a shaking-out method. Schunck's observations were near the truth but can not be compared in accuracy with the method of separation which Tswett was able to develop from the colloidal properties of xanthophylls.

Tswett hit upon a very ingenious method indeed of applying the results of his study. He filtered the moisture-free petroleum ether solution of the mixed chloroplastid pigments (or carbon disulfide solution) through a column of perfectly dry $CaCO_3$, packed as tightly and evenly as possible in a glass tube, and found that the various pigments differentiated themselves according to their adsorption affinity (colloidal aggregation) for the $CaCO_3$. The resulting chromatogram (as Tswett proposed to call it) presented a most surprising picture of the chloroplastid pigments, which is strikingly similar in effect, if not in principle, to the well-known Liesegang phenomena.

By applying this chromatographic method of analysis to petroleum

ether and carbon disulfide solutions of the chloroplastid pigments from plantain (*Plantago*) and dead nettle (*Lamium album*) leaves Tswett has shown that at least three and possibly four xanthophylls accompany carotin. He has provisionally designated these α, α', α'', and β xanthophylls, respectively. Tswett has characterized these pigments further, as follows:

Xanthophyll α. This pigment is least adsorbed by the $CaCO_3$ and is closest to carotin in this respect, which is not adsorbed at all. Its adsorption zone is the lowest in the column of the xanthophyll zones and has an orange-yellow color when carbon disulfide is the solvent. It is hypophasic in the Kraus separation, i.e., remains in the alcohol layer. It shows three well marked absorption bands, the first two of which, in alcohol or petroleum ether solution, lie at 485-470μμ and 455-440μμ. Its alcoholic solutions are merely bleached on addition of con. HCl.

Xanthophylls α' and α''. These pigments lie very close together in the column but above the zone of xanthophyll α. In CS_2 their zones are yellow. They are similar in properties to xanthophyll α, i.e., in the Kraus separation and spectroscopically, but their absorption bands are shifted slightly towards the violet. The effect of HCl on the alcoholic solutions is not mentioned but the author (1914g) has found that for xanthophyll α', at least, no color reaction is produced.

Xanthophyll β. This pigment shows the greatest adsorption affinity for $CaCO_3$ (exists in the largest colloidal aggregates) and comprises the highest yellow zone in the column. This pigment is hypophasic in the Kraus separation like the other xanthophylls, but may be differentiated from them by the fact that its alcoholic solution gives a blue color on addition of con. HCl, and also by the fact that its absorption bands are shifted perceptibly towards the violet from those of xanthophylls α, α', and α'', the first two bands lying at 475-462μμ and 445-430μμ, when in alcoholic solution. The xanthophyll β of Tswett appears to be identical with the "yellow xanthophyll" of Sorby and the Y. xanthophyll of C. A. Schunck, but bears no relation whatever to the xanthophyll β of Kohl. According to Tswett (1908b) the latter is not a xanthophyll at all, in fact does not exist in the plant but is merely a post-mortem decomposition product derived from colorless chromogens whose alkali salts are yellow and which assume a dark color on oxidation.

The relative solubility properties of carotin and xanthophylls as

exhibited in the Kraus separation indicated to Tswett (1906a) a fundamental chemical difference between the two groups of carotinoids. The proof of this theory as well as the nature of the difference was soon brought to light by Willstätter and Mieg (1907) when they isolated the first crystalline xanthophyll and submitted it to analysis. Working on the same elaborate scale, which has characterized all the researches on carotinoids in Willstätter's laboratory, a crystalline xanthophyll was isolated from 100 kilos of dried nettle (*Urtica*) leaves. The average of five ultimate analyses of crystals prepared both by recrystallization from methyl alcohol and from chloroform (by addition of petroleum ether) showed 84.22 per cent carbon and 9.92 per cent hydrogen, which corresponds very closely with the theoretical values of 84.44 per cent carbon and 9.93 per cent hydrogen for the formula $C_{40}H_{56}O_2$. This was confirmed fairly well by a molecular weight determination (found 512, theory 564), and better by an analysis of the iodine content of the theoretically simplest iodine addition product, $C_{40}H_{56}O_2I_2$ (found 31.68 per cent, theory 30.86 per cent).

The chemical properties of the crystalline xanthophyll isolated by Willstätter and Mieg will be considered in detail elsewhere. Several points, however, may profitably be considered at this point. The crystalline product showed the greatest solubility difference from carotin in alcohol and low boiling petroleum ether, being practically insoluble in the latter, but readily soluble in the former, which is just the reverse of carotin in these solvents. The Kraus method of separation of the pigments was further confirmed by Willstätter and Mieg by applying the test in several ways to solutions of the purified pigments. The difference between the position of the absorption bands of carotin and the xanthophylls, first pointed out by Monteverde, was confirmed, the first two bands as measured by Willstätter and Mieg lying at 480-470$\mu\mu$ and 453-437$\mu\mu$.

Willstätter and Mieg expressed their belief in the existence of a group of xanthophylls in the paper under consideration although they were apparently not familiar with Tswett's demonstration of this fact a year before their paper appeared. The question naturally arises as to which xanthophyll was obtained in crystalline form by these investigators.

Tswett (1910a) has expressed the opinion that the xanthophyll crystallized by Willstätter and Mieg was a mixture of two or three xanthophylls in which xanthophyll α predominated, a possibility which was later acknowledged by Willstätter and Stoll (1913b). The evi-

dence available on this question indicates, however, that xanthophyll β may have formed a considerable proportion of the crystalline preparation. Willstätter and Mieg mention the fact that their preparation dissolved in strongly alcoholic HCl with a blue color, a reaction which is apparently characteristic of xanthophyll β only. In the Sorby and C. A. Schunck separation, however, the pure pigment differentiated itself almost equally between the alcohol and carbon disulfide layers, a reaction which obviously characterizes the α group of xanthophylls because of their lesser adsorption from this solvent by $CaCO_3$. Still further evidence of a mixture of xanthophylls in the Willstätter and Mieg preparation is the fact that its spectroscopic absorption bands apparently lie in an intermediary position between the bands of xanthophylls α and β as recorded by Tswett.

The isolation of the various members of the xanthophyll group in crystalline form seems greatly to be desired in order that the differences existing between the individual members of this class of carotinoids may be determined. The relative adsorption properties of these pigments offers the most promising method for accomplishing this result but the experimental work would have to be conducted on a very generous scale. The xanthophylls are unquestionably either isomorphic or isomeric forms of the same empirical composition, $C_{40}H_{56}O_2$, as Willstätter and Stoll (1913) have pointed out. The author believes that Willstätter and Escher (1912) have already isolated pure xanthophyll α in the form of their so-called lutein from egg yolk, as will be discussed more fully in a later chapter.

It is not likely that more than four xanthophylls characterize the chloroplastid for the author (1914g) has found only four on applying the chromatographic method to extracts from an entirely different plant than Tswett used, namely, the leaves of alfalfa (*Medicago sativa*). The possibility of other xanthophylls being present in nonchlorophyllous organs is indicated, however, by a chromatographic analysis which the author (1914g) carried out on the xanthophyll fraction (obtained by the Kraus separation) of the pigments of the carrot root, in which no less than eight distinct yellow or orange zones characterized the chromatogram. The possibility remains to be investigated, however, whether this result was influenced in any way by the method of preparation of the material or other experimental steps in the procedure employed. The author regards the adsorption phenomenon of the carotinoids as colloidal so that it may not be possible to secure these pigments in every case in the same

degree of colloidal aggregation. Willstätter and Mieg found that their crystalline xanthophyll readily entered into combination with solvents forming molecules of solvent of crystallization, which is unquestionably a colloidal combination and might easily influence greatly the adsorption properties of the pigments. The whole adsorption phenomenon deserves a further study using pure preparations of the individual pigments.

The xanthophylls are usually regarded as pigments in which yellow is the predominating color. Red colored xanthophylls also exist, however. Monteverde (1893) first called attention to a red pigment in the reddish-brown leaves of the young floating pond weed (*Potamogeton natans*), an aquatic herb widely distributed in Russia, which showed the xanthophyll properties in the Kraus separation. This pigment has since been called rhodoxanthin by Monteverde and Lubimenko (1913b), who obtained it in crystalline form. The pigment appears to be isomeric with the xanthophyll of the chloroplastids, as lycopin is isomeric with carotin. It differs from the usual yellow xanthophyll by dissolving in formic acid with a yellow color, yellow xanthophyll dissolving in this solvent with a green color, according to Monteverde and Lubimenko. Rhodoxanthin also shows spectroscopic absorption bands with characteristic position, especially in carbon bisulfide. A comparison of the xanthophyll and rhodoxanthin bands in this solvent, as given by Willstätter and Stoll (1913) and by Monteverde and Lubimenko, respectively, is shown in the following:

	Xanthophyll (W. and S.)	*Rhodoxanthin* (M. and L.)
Band I	516–501 μμ	575–553 μμ
Band II	483–467 "	535–515 "
Band III	447–441 "	500–480 "

The general solubility properties of rhodoxanthin appear to follow those of xanthophyll very closely.

The relation between the empirical constitution of carotin and the xanthophylls is such that the latter may be expressed very simply as carotin dioxides. The character of the oxygen combination, however, is not clear, for according to the statement of Willstätter and Mieg their crystalline xanthophyll did not show the presence of either hydroxy, carboxyl or carbonyl groups. The xanthophylls, therefore, cannot be simple oxidation products of carotin. But these statements regarding the character of the oxygen in the xanthophyll molecule possibly should be confirmed, for notwithstanding the fact that it has not yet been found possible to transform carotin into xanthophyll in

the laboratory, the constant presence of these pigments in the chloroplastid is very difficult to explain unless the constitution of one type of pigment bears a simple relation to that of the other. The various theories which have been offered regarding the possible functions of the carotinoids in the chloroplastid also fall down unless the carotinoids are closely relationed chemically. Ewart (1915), to be sure, has recently claimed to have succeeded in reducing xanthophyll to carotin in the laboratory. The evidence for this is very unconvincing, especially in view of the fact that Ewart on subsequent study (1918) failed to substantiate any of the other products which he first claimed to have produced from xanthophyll on photo-oxidation. The reduction experiment of xanthophyll to carotin unfortunately was not repeated in the second study.

Carotinoids in Etiolated Leaves

The yellow chromolipoids which develop without chlorophyll in the leucoplastids, when plants are grown in the dark, would seem to be closely related to, if not completely identical with those found in the chloroplastids, at least qualitatively, inasmuch as etiolated plants form chlorophyll very rapidly in the light without loss of yellow constituents in the resulting chloroplastids. No studies have been made of the pigments of etiolated leaves, however, since our newer chemical conceptions of the plant carotinoids have arisen so that it is necessary to depend upon older investigations for our experimental knowledge of these coloring matters. It is possible to state with certainty that carotin is present in the etiolated plant, but the evidence is insufficient to substantiate the belief of Tammes (1900) and Kohl (1902f) that it is probably the only yellow chromolipoid present inasmuch as it is now known that the methods which these investigators employed are not specific for carotin.

Joannes Rajus (1693) appears to have first recorded the observation that plants which grow in the dark do not turn green but have a yellow color. Bonnet (1754) named such plants "plantes étiolées." The question whether the yellow pigment or some colorless substance was the forerunner of the green pigment which developed so rapidly when etiolated plants are exposed to the light occupied the attention of many investigators. Interest in this question was stimulated by the discovery of Phipson (1858) that etiolated leaves rapidly assume an emerald green color when immersed in con. H_2SO_4. Phipson followed

the terminology of Berzelius for yellow autumn pigments and called the etiolated pigment xanthophyll.[3] Frémy (1860) naturally regarded the yellow pigment of young sprouts and etiolated leaves as identical with his phylloxanthine and the bluish-green color which develops on treatment with acid (he also found the fumes of HCl and HNO_3 very effective) as identical with his phyllocyanine. Sorby (1871b) recognized the relation of the yellow pigment of etiolated leaves to other yellow plant pigments and regarded the color as due to a preponderance of his so-called "xanthophyll" group, characterized by their solubility in carbon disulfide and two more or less distinct spectroscopic absorption bands in the blue part of the spectrum. Gregor Kraus (1872b) compared the spectroscopic properties of the alcoholic extract of etiolated leaves with his xanthophyll pigment which remained in the alcohol on shaking leaf extracts with benzene. The results led him to believe that the pigments were probably identical, and he proposed a genetic relation of the etiolated pigment to the green pigment of plants.

Pringsheim (1874), however, also using a spectroscopic examination of the alcoholic extracts of etiolated leaves as the basis of his conclusions, found characteristic absorption bands in the red end of the spectrum in addition to bands in the blue which characterized Kraus' xanthophyll. Inasmuch as the same result was obtained for each of 10 different etiolated plants which he examined, Pringsheim concluded that a special pigment was present which caused the bands in the red as well as the bands in the blue. He called this pigment etiolin. Pringsheim's results have been frequently substantiated, and while some subsequent investigators (Wiesner (1877b), Elfving (1882), Tschirch (1884)) have agreed with his conclusion regarding etiolin as a distinct pigment, a majority (Timiriazeff (1875), Hansen (1884b), Immendorff (1889), Monteverde (1894), Kohl (1902f), Greilach (1904) who have studied this phase of the etiolin question have presented convincing evidence that the spectroscopic absorption bands in the red which Pringsheim observed in his etiolated leaf extracts are

[3] According to Czapek (Biochemie der Pflanzen, 2nd Ed., vol. I, p. 579, Jena, 1913), Julius Sachs (1859 a, b) and Jos. Boehm (1859) called the etiolated pigment leukophyll and chlorogon, respectively. The statement is incorrect. The leukophyll of Sachs was a colorless chromogen in the seeds and also in the etiolated plants which gave rise to the green chlorophyll in the sunlight or on treatment with acids (compare Phipson [1858]), while the chlorophor (not chlorogon as Czapek has it) of Boehm was the same colorless chromogen. Boehm differed from Sachs in regarding the green acid derivative of the colorless chromogen as an artificial pigment and the green sunlight derivative as the true chlorophyll. Both investigators recognized the existence of the yellow etiolated leaf pigment as well as the colorless chromogen.

either chlorophyll or a closely related forerunner of one of the chlorophyllins. Timiriazeff (1875) believed the absorption spectra of alcoholic etiolated leaf extracts to be due to a small amount of chlorophyllin admixed with Kraus' xanthophyll. Hansen (1884b) regarded the bands in the red as due to chlorophyll. Monteverde (1894) regarded the substance giving the bands in the red as a forerunner of one of the chlorophyllins and called it protochlorophyll, a view which seems to have been substantiated by the work of Greilach (1904). The latter proposes to reserve the name etiolin for this green pigment with properties like chlorophyll which exists in etiolated leaves in very small amounts, and to use the term in the same sense as Monteverde used the word protochlorophyll. According to Greilach etiolin (protochlorophyll) is not a constant constituent of the etiolated leaf but appears and then disappears during the germination of the seed in the dark.

Arnaud (1889), following his earlier (1885) demonstration regarding the identity of the yellow leaf pigment isolated by him with the carotin from carrots, regarded the yellow color of etiolated leaves as due to the same pigment. No chemical proof was offered of this but he determined the quantity of carotin in the etiolated leaves of the kidney bean (*Phaseolus vulgaris*), using a colorimetric method which will be reviewed in a later chapter. Inasmuch as Arnaud's method of analysis would preclude all but traces of xanthophylls his result may be regarded as the first proof of the presence of carotin in etiolated leaves. This was confirmed completely by Immendorff (1889) the same year. He saponified the alcoholic extracts from etiolated leaves, extracted the carotinoids from the soap with ether and obtained crystals of carotin from the golden yellow extract. He did not succeed in obtaining crystals from etiolated leaves which had developed only a pale yellow color, but only from those having a more orange color, but this cannot be interpreted as indicating another pigment in the less pigmented leaves, as Immendorff believed, but must be regarded as due solely to differences in concentration of pigment, as Kohl (1902f) has pointed out.

Following Immendorff, Molisch (1896), Tammes (1900) and Kohl (1902f) have independently substantiated the presence of carotinoids in etiolated leaves from various plants using microscopic crystallization methods on the fresh tissues. Inasmuch as our information regarding the yellow chromolipoids in the etiolated leaf depends at the present time on the observations of these authors and the microchemi-

cal methods which they used, it will be necessary to state briefly the character and significance of the methods, reserving a fuller description for a later chapter.

Frank (1884) first observed that red crystalline needles form in the plastids and between the chlorophyll granules when green leaves are immersed in dilute acids for a time, and then, after washing off the acid, are allowed to remain in distilled water for a still more protracted period. Tschirch (1884), who first examined the phenomenon, did not decide the nature of the crystals, but Molisch (1896) found the crystals to be identical in properties, although having a more reddish color, with the majority of crystals which he found could be produced by an entirely different method. Molisch's method is to immerse the leaves in dilute (40 per cent by volume) alcohol containing 20 per cent KOH, until the chlorophyll is completely extracted. The process sometimes requires several days. On washing off the green extract with water, and immersing the washed leaves in distilled water for several hours to insure the complete removal of the chlorophyll, it is found that crystals of various forms and colors from yellowish-orange to red have appeared abundantly in the leaf. Molisch proved fairly conclusively the identity of many of the crystals thus obtained with the red-orange crystals which form in concentrated alcoholic leaf extracts, and accordingly decided to call the crystals carotin. Molisch was careful to point out, however, that he used the term carotin in the sense of a group of closely related pigments, for he recognized that the crystals formed by his alkali method were not due in all cases to the same pigment. Tammes (1900) and Kohl (1902), however, who greatly extended our knowledge of the presence of carotinoids in the plant kingdom, using the microchemical methods of Frank and Molisch, believed that only one pigment was concerned, namely, carotin, and regarded the methods as specific for this pigment. Tswett (1911a), however, proved definitely that the crystals obtained by Molisch's method are a mixture of carotinoids, and this has been completely confirmed by van Wisselingh (1915). Other microchemical methods for carotinoids have been worked out by the two investigators just mentioned, and these will be reviewed in a later chapter. Tswett has stated that Frank's acid method may possibly be specific for carotin. This may well be the case in view of the much greater sensitiveness of the xanthophylls to acids, as van Wisselingh has pointed out, but this investigator who has studied the method closely finds it to be often laborious, requiring sometimes several months for

the crystals to form, and that it frequently fails to show the presence of carotin in plant tissues in which the pigment is known to be present. By the use of suitable solvents van Wisselingh has demonstrated very ingeniously, however, that it is possible to distinguish the xanthophyll crystals as a group from the carotin crystals in the mixture formed by the Molisch method in various plant tissues.

Returning now to the investigations regarding the chromolipoids in various etiolated plants, it may be stated that Molisch (1896) demonstrated carotinoids by his alkali method in the etiolated leaves of garden cress (*Lepidium sativum*), barley (*Hordeum vulgare*), hemp (*Cannabis sativa*), oats (*Pisum sativum*), and of balsam (*Balsamina hortensis*, D.C.) and fir (*Abies excelsa*), but not from the sunflower (*Helianthus annuus*), in which only orange-red drops formed. Tammes (1900), using seeds from the same plants, save the balsam and fir, substantiated Molisch's positive results with the alkali method, and in addition obtained the carotinoid crystals in the etiolated di-cotyledons of the sunflower. Kohl (1902f) denounced most emphatically the view of Pringsheim that a specific pigment is present in etiolated leaves, devoting an entire chapter of his monograph to this question. In addition to the etiolated plants examined by Molisch and Tammes, Kohl (1902f) was able to form carotinoid crystals in the etiolated leaves of the turnip (*Sinapis alba*) and various varieties of Asphodel.

It is apparent that we have as yet only indirect evidence that xanthophylls are present in the etiolated leaf. C. A. Schunck (1903) has furnished direct evidence of xanthophylls in an isolated case, namely, the etiolated leaves of the daffodil (*Narcissus pseudo-narcissus*), using the carbon disulfide separation method which has already been described. A mixture of xanthophylls was found to be present, but Schunck was unable to obtain crystals of chrysophyll (carotin) although he had no difficulty in obtaining them abundantly from alcoholic extracts of the etiolated leaves which had been allowed to turn green in the sunlight. Greilach's (1904) spectroscopic observations of the pigments in etiolated leaves led him to conclude that yellow pigments other than carotin are also concerned in the coloration of the leaves. Ewart (1918) states that he has found 8 to 10 parts of carotin to one of xanthophyll in etiolated wheat seedlings.

Several investigators have studied the question raised by the last observation of Schunck, namely, what effect greening has on the content of carotinoids in the etiolated leaf. Wiesner (1877a) first studied this point and concluded that the xanthophyll (carotinoids) diminished

during the greening of etiolated sprouted oats. Inasmuch, however, as he used a colorimetric comparison of the total alcoholic extract of the etiolated plant with the alcoholic xanthophyll layer of the extract from the green plant following the Kraus separation, it is not difficult to account for his results. Arnaud's (1889) quantitative colorimetric comparison of the carotin content of etiolated and green leaves of the kidney bean, referred to above, led to completely opposite results. Arnaud's data (calculated from his colorimetric reading) show 34.0 mg. carotin in 100 grams of the dry etiolated leaves, and 178.8 mg. in the same amount of dry green leaves, a result which appears to have been substantiated by the observation of Schunck on etiolated and green daffodil leaves. Kohl (1902f) studied the same question and drew the same conclusion as did Arnaud, namely, that carotin increases during greening. His method of analysis, however, does not permit so exact an interpretation, for he merely compared colorimetrically the total unsaponifiable pigment extracted from the leaves by alcohol. Kohl's carotin solutions were thus a mixture of carotin and xanthophylls. It is not possible to decide from these observations whether xanthophylls as well as carotin increase in the etiolated leaf during greening. This appears to be the case, however, in view of Ewart's (1918) statement, quoted above, and the fact that xanthophylls are the predominating carotinoids in green leaves as found by Willstätter and Stoll (1913) and Miss Goerrig (1917).

An interesting phase of the etiolated leaf pigmentation is that of the most favorable conditions for the development of the carotinoids. Light and temperature are obviously the controlling factors. Wiesner (1877b) observed that potato sprouts, which formed in the light, showed little if any yellow pigment, while those which formed in the dark developed from 30 to 150 per cent more pigment. More interesting is the result of Elfving (1882), which was confirmed by Immendorff (1889), that carotinoids increased greatly in leaves under conditions which depressed chlorophyll formation, i.e., low temperatures (2° to 8° C.) and very diffused light.

Carotinoids in Naturally Yellow Leaves

Plastids which fail to develop chlorophyll but in which other pigments form instead are called chromoplastids. The pigments of chromoplastids are usually granular, sometimes crystalline and almost invariably yellow to red in color. In the case of some plants the leaf

plastids are always characterized by an absence of chlorophyll, the leaves being yellow or golden yellow in color. Several investigators have studied the pigmentation of such plants in relation to the yellow chromolipoids which characterize the chloroplastid. Thudichum (1869), many years ago, observed the relation between the pigment of the carrot root and that in the yellow leaves of *Coleus,* and included them both in his group of luteins. Dippel (1878) found that the spectroscopic absorption bands of the pigment extracted by alcohol from yellow leaves corresponded with those of the yellow pigment which he found to be present in Kraus' cyanophyll layer from green leaf extracts. Dippel called this pigment xanthin.

Tammes (1900) and Kohl (1902g) first sought to show the relation of the pigment of such leaves to carotinoids. Tammes found that the plastids of yellow leaves gave positive carotinoid reactions with con. H_2SO_4, con. HNO_3, and with HCl containing phenol and with bromine water, when the leaves were first dried. Yellow leaves in which the chromoplastids had disintegrated failed to give these reactions. When examined after submitting the leaves to the Molisch alkali crystallization method brownish yellow crystals of various shapes were observed in each of the following cases:

1. *Aucuba japonica* Thunb. (Japanese Aokiba).
2. *Elaeagnus latifolia* L.
3. *Euonymous japonicus* L. variety *sulphurea* (Japanese spindle tree).
4. *Sambucus nigra* L., variety *aurea.* (European elder).

Kohl (1902g) substantiated these results, also using the Molisch method, and in addition obtained carotinoids in the following plants with naturally yellow leaves.

1. *Abutilon nervosum* (Flowering maple).
2. *Betula* species (Birch).
3. *Spiroea* species, variety *aurea.*

Kohl made a more exhaustive study of the pigment of yellow elder leaves (*Sambucus nigra foliis luteis*) and found that the dilute alcoholic KOH extract of the leaves gave up only a small part of the pigment to ether. The ether extract thus obtained gave the carotin spectrum, but the pigment remaining in the alkaline alcohol layer showed only end absorption of the spectrum. He believed that this pigment was due in part to the β xanthophyll of Tschirch (1887), and in part also to a new pigment which he called phyllofuscin, which differs from β xanthophyll in being partly extracted from its aqueous solution by ether. It seems likely that Kohl was dealing here with

Tswett's (1908b) group of colorless chromogens, whose alkali compounds are characterized by a dark yellow or brown color. No xanthophyll pigment showing spectroscopic absorption bands is present in leaves having yellow plastids, according to Kohl.

Van Wisselingh (1915) made some microscopic observations of the crystals which he produced in the yellow spotted leaves of *Croton ovalfolius* Vahl., *Graptophyllum pictum* Griff. (caricature plant), and *Sambus nigra* L. *foliis* var., using both the alkali method of Molisch and the acid method of Frank. The crystals produced by the latter method are described by van Wisselingh as brown crystal aggregates, the yellow spots in the leaves of *Sambus nigra* showing reddish colored crystals also, and the similar spots in the leaves of *Graptophyllum pictum* showing orange-red platelets and small orange-yellow crystal aggregates.

Summarizing our knowledge of the pigments in naturally yellow or yellow spotted leaves, it seems certain that carotinoids are concerned in part in the pigmentation, but the information is lacking regarding the types and distribution of the carotinoids between carotin and xanthophylls. It also remains to be determined whether water-soluble pigments of the type whose alkali salts are golden yellow in color, which are probably related to flavones, are, as Kohl believed, normally a part of the pigmentation.

Carotinoids in Yellow Autumn Leaves

The hidden beauties of the forest, revealed in the passing of the chlorophyll of the leaves in the autumn, were among the earliest to attract the attention of the plant chemists. The author has not had an opportunity to determine how far back scientific observation on the autumn leaf pigment can be traced. Guibourt, however, as early as 1827, expressed the belief that the yellow and red pigments of autumn leaves were due to a coloring matter which persisted after the green chromula of the leaves had disappeared. Guibourt observed that those families whose flowers and fruits were characterized by yellow pigments had yellow leaves in the autumn, while the families with red colored flowers and fruits had red autumn leaves.

Macaire-Prinsep (1828) apparently made the first chemical examination of the autumn pigments. He found that the yellow pigment of the autumn leaves of the Lombardy poplar (*Populus fastigiata*) could be extracted with ether or hot alcohol; that the pigment thus extracted

turned green on treatment with alkalis; that a yellow autumn leaf recovered its green color when immersed in alkali, while green leaves turned yellow and finally red when treated with acids. He accordingly proposed the term polychrome for the green chromula of the leaf which thus changes from green to yellow to red and *vice versa,* and regarded the phenomena which he observed as the exact duplication of the yellow and red autumn coloration of leaves. Berzelius (1837a), a little later extracted the yellow color from the autumn leaves of the pear tree (*Pyrus communis*) with alcohol (about 85 per cent), and called the pigment xanthophyll. Berzelius noticed that the pigment readily bleached, but regarded it as a fat and as being derived from the green pigment of the leaf. He was careful to distinguish (1837b) between the pigment xanthophyll and the red pigment which could be extracted from the red autumn leaves of the mountain ash (*Sorbus aucuparia*) and cherry trees (*Prunus cerasus*), and from the red autumn leaves of gooseberry (*Ribus glossularia,* var. *rubra*) and common barberry (*Berberis vulgaris*) bushes, which pigment Berzelius called erythrophyll.

Following these very early experiments which were carried out before the development of our present-day knowledge of the yellow chromolipoids one finds a number of more or less unrelated observations regarding the yellow autumn colors, which were made incidental to some of the plant pigment studies which have already been reviewed in connection with the carotinoids in the chloroplastid. For example, Frémy (1860) regarded the autumn coloration as due to his phylloxanthine, the isolation and properties of which were described in an earlier paragraph. As already pointed out, Frémy believed that two pigments existed in green leaves, a greenish-blue phyllocyanine and a yellow phylloxanthine, and that the autumn colors were the result of the fact that the latter pigment was more stable than the former. Sachs (1863) made a microscopic study of leaves during the autumn color change and proposed the theory that the chlorophyll migrates out of the plastids leaving behind a larger number of intensely yellow granules. The latter were actually observed and were soluble in alcohol. Mer (1873), however, was not able to substantiate this belief regarding a migration of the green granules out of the leaf cells, but the presence of yellow or brownish-yellow plastids in the cells of the autumn colored leaves, at least in the necrobiotic [4] phase, is well

[4] According to Tswett (1908 c) the autumn coloration occurs in two phases, namely, the necrobiotic and the postmortal. The former is always characterized by a yellowing

established (Tammes (1900), Goerrig (1917)). True chromoplastids are, in fact, present.

The disappearance of chlorophyll from the leaves in the autumn is alone sufficient evidence of vital changes taking place in the protoplasm. Viewing the phenomenon of autumn coloration, therefore, in the light of what is now known regarding the yellow chromolipoids of the chloroplastids the questions naturally raised, as recently pointed out by Miss Goerrig (1917) are: (1) are the autumn pigments merely the yellow carotinoids already present in the chloroplastids, (2) are the natural carotinoids of the green plastids augmented or possibly replaced by other yellow pigments which may be closely related but still capable of being differentiated from the normal carotinoids, or (3) are the autumn pigments entirely new substances which replace the normal carotinoids, destroyed, perhaps, with the chlorophyll? In spite of the fact that these questions have been studied by Miss Goerrig (1917) and by Tswett (1908b) using the most modern methods, the question of autumn coloration, at least with respect to the yellow colors, is not yet entirely cleared up. One can apparently state definitely that the yellow colors are not due to entirely new pigments. Whether the plastid carotinoids are present unchanged or slightly modified is not so certain. It is also uncertain to what extent yellow pigments of an entirely foreign nature are present and what part they play, if any, in the autumn coloration.[5] Tswett's and Miss Goerrig's studies differ decidedly on this point.

We know from Miss Wheldale's (1916) splendid monograph that there is a large group of trees, shrubs and climbing plants in whose leaves red anthocyanins form abundantly in the autumn. Some of those mentioned by Miss Wheldale have been shown by certain investigators to contain carotinoids also. Other plants do not form anthocyanins in their foliage in the autumn. Miss Wheldale mentions a number of the latter in which autumn carotinoids have not yet been demonstrated. These three groups of plants, together with the names of the investigators who have demonstrated carotinoids in the autumn leaf, are summarized in Tables 1, 2 and 3.

of the plastids and a retention of the osmotic pressure of the cell plasma. The latter is recognized by the disappearance of the plastid pigments, the disintegration of the protoplasm and the formation of brown, reddish brown or black pigments as the result of an oxidation of colorless, water-soluble chromogens.

[5] In isolated cases such as the yellow leaves of the osage orange (*Maclura aurantiaca*) one no doubt finds an abundance of the characteristic yellow flavones, morin and maclaurin found in the wood of this plant (Kressmann, 1914) in addition to carotinoids (Goerrig, 1917).

Table 1. Autumn Foliage Containing Both Anthocyanins and Carotinoids

Acer campestris (Common [illegible] Maple), Tammes, Tswett, Goerrig.
Acer platanoides (Norway [illegible]), Tswett, Goerrig.
Acer [illegible] (Sycamore), Arnaud in green leaves.
Aesc[illegible] (Buckeye), Tammes, Tswett, Goerrig.
Crataegus pinnatifida (Hawthorne), Tswett.
Hedera helix (English Ivy), Arnaud in green leaves.
Prunus avium (Sweet Mazzard Cherry), Tammes.
Pyrus germanica (German Pear), Tswett.
Pyrus ussuriensis (Pear), Tswett.
Quercus rubra (Red Oak), Staats.
Rosa rugosa (Rugosa rose), Tswett.
Taxodium distichum (Bald Cypress), Goerrig.
Vitis Coignetiae (Grape), Goerrig.

Table 2. Autumn Foliage Containing No Anthocyanins

Alnus glutinosa (Black Alder).
Aralia (Ginseng), Tswett.
Betula alba (White Paper-birch), Staats.
Broussonetia papyrifera (Paper Mulberry), Goerrig.
Carpinus Betulus (European Hornbeam).
Castanea (Chestnut).
Celastrus scandens (Climbing Bittersweet), Tammes.
Convalaria majalis (Lily-of-the-valley), Tswett.
Corylus Avellana (Hazelnut).
Dioscora batatas decn. (Yam), Tammes.
Fraxinus excelsior (Ash).
Funkia Sieboldii, Tswett.
Ginkgo biloba (Maidenhair tree), Tswett, Goerrig.
Gleditsia triacanthos (Honey Locust), Tswett.
Iris germania (Fleur-de-lis or Iris), Tswett.
Juglans regia (English Walnut).
Larix europaea (European larch), Tswett.
Lepidium Draba (Pepperwort), Goerrig.
Liriodendron tulipifera (Tulip tree), Tswett.
Maclura aurantiaca (Osage Orange), Goerrig.
Mirabilis Jalapa (Four-o'-clock), Goerrig.
Morus alba (Mulberry), Arnaud from green leaves.
Platanus orientalis (Oriental sycamore), Goerrig.
Polygonum sachalinense (Sacaline), Goerrig.
Populus alba (Silver Poplar).
Populus nigra (European Black Poplar).
Populus tremula (Aspen Poplar), Tswett.
Ptelea trifoliata (Hop tree), Tswett.
Pyrus communis (Common Pear), Berzelius.
Quercus (Oak), except *Quercus rubra* and *Q. coccinea*.
Quercus Robur (English Oak), Tammes.
Rhus toxicodendron (Poison Ivy), Tswett.
Salix babylonica (Weeping Willow), Goerrig.
Salix Caprea (Goat Willow), Goerrig.
Sparmania africana, Tswett.
Ulmus campestris (Elm), Immendorff, Tammes, Goerrig.

Table 3. Red Winter Foliage Due to Carotinoids

Aloës, Molisch.
Conifers, Lubimenko.
Cryptomeria japonica, Tswett.

Cupressus Naitnocki (Cypress), Tswett.
Encephalartos Hildebrandtii A. Br. and Bouche, Lubimenko.
Juniperus virginiaca (Red Cedar), Tswett.
Macrozamia species, Lubimenko.
Retinospora plumosa (Juvenile Thuja), Tswett.
Selaginella (Club Moss), Molisch, Lubimenko.
Taxus baccata (Yew), Tswett.
Thuja orientalis (Arbor Vitæ), Tswett, Monteverde and Lubimenko.

Opinion was divided even among the older investigators as to whether one or several pigments are involved in the autumn coloration, and as to what relation they bear to the normal pigments of the green leaf. Among those who believed that only one pigment is involved may be mentioned Pringsheim (1874), whose spectroscopic observations of extracts of yellow autumn oleander leaves and rye straw showed three absorption bands in the blue only. Pringsheim concluded that the pigment involved was different from his etiolin, and he adopted Berzelius' name of xanthophyll for the yellow autumn pigment. Tschirch (1884) also believed that only one pigment exists in yellow autumn leaves, which he called β xanthophyll, to distinguish it from an α xanthophyll, the yellow pigment in green leaves, although he regarded the two xanthophylls as closely related, if not identical. Tschirch believed that there was less xanthophyll in autumn leaves than in green leaves. The pigment thus described by Tschirch was probably carotin. Immendorff (1889) succeeded in obtaining carotin crystals from alcoholic extracts of the yellow autumn leaves of the beech (*Fagus*) and elm (*Ulmus campestris*), and although he admits that he secured a very small quantity and that only in one case, his extracts always showed the carotin spectrum, which caused him to conclude that carotin is the cause of the yellow autumn coloration. Tammes (1900) examined the fallen autumn leaves of a number of trees and shrubs after submitting them to the Molisch alkali crystallization method. The plants examined are given in Tables 1 and 2. Carotinoid crystals were observed in all cases in which the leaves still showed the presence of yellow plastids. Tammes' conclusion that carotin is the cause of the yellow autumn coloration is, of course, not valid, in view of the fact that we now know that the Molisch reaction is not specific for carotin.

In view of the multiplicity of carotinoids at present acknowledged to exist in the chloroplastids the idea of only one pigment in the yellow autumn coloration is not acceptable. A number of the older investigators concluded that more than one pigment was involved even

before it was definitely known that several yellow chromolipoids exist in the green leaf. For example, Gregor Kraus (1872c) believed that the yellow autumn pigment was due in part to his yellow xanthophyll and in part to a yellow water-soluble pigment. Sorby's (1871a, b) idea that autumn coloration is due to varying mixtures of xanthophylls, erythrophylls and chrysotannins is not far different from Miss Goerrig's recent conclusion when one is acquainted with the particular properties of Sorby's pigment groups. Sorby's xanthophylls are our present carotinoids; his erythrophylls are acknowledged to be our red anthocyanins (they were characterized by being strikingly affected in color by alkalies and acids); and his chrysotannins, which he believed increased during the autumnal color changes, were indefinite water-soluble yellow pigments with acid properties (related to tannic acid) which readily deepened in color on oxidation. Miss Goerrig found abundant quantities of a yellow pigment in autumn leaves, which could be extracted with dilute acetone. Saponification of the extract greatly intensified the color, and the unsaponified pigment could not be extracted from the dilute acetone by ether. There also seems to be little reason to doubt the identity of Sorby's chrysotannin and Miss Goerrig's unnamed yellow water-soluble pigment with the so-called autumn-xanthin which Staats (1895) extracted with alcohol from the yellow autumn leaves of the linden (*Tilia*), beech (*Fagus*), ash (*Fraxinus*) and red oak (*Quercus rubra*), and which he obtained in the form of a red crystalline water-soluble potassium salt. Staats ascribed the autumn pigmentation solution to this coloring matter, but in this he was, of course, mistaken. The alcoholic extract of the oak leaves first turned green and then yellow with the precipitation of the potassium salt, when treated with KOH, confirming the observation of Macaire-Prinsep (1828), mentioned above, on extracts from autumn poplar leaves. The explanation of this interesting color reaction of autumn leaves is not apparent.

Carl Kraus (1875) also ascribed the autumn coloration to more than one pigment, naming two, xanthin, especially, and also xanthophyll. To explain briefly his terminology it may be stated that his xanthophyll was practically the Gregor Kraus xanthophyll in that author's alcohol-benzene separation, Carl Kraus characterizing it further because of its change to a blue pigment on treatment with acid (this is either the phylloxanthine reaction of Frémy (1860), or the xanthophyll β color reaction of Tswett (1911a)). The xanthin of Carl Kraus, however, is undoubtedly carotin, since he found it in the

benzene layer of the Gregor Kraus separation, and it was not turned blue by acids.

C. A. Schunck's (1903) splendid spectroscopic observations of the xanthophylls included an examination of the pigments of yellow autumn leaves. The method of preparation of the material for these studies, which has already been described, precludes the presence of pigments other than carotinoids in the alcoholic solution submitted to Schunck's carbon disulfide separation. Schunck does not mention chrysophyll (carotin) in connection with his autumn leaf examination, but his xanthophyll solution gave four banded spectra in practically all cases. The conclusion drawn was that autumn coloration is due to L. xanthophyll and a preponderance of the acid derivative of B. xanthophyll, which is characterized by a four banded spectrum, a result which strongly supports the idea that certain changes do occur among the carotinoids of the leaf during the necrobiotic period.

Kohl (1902h) made a careful study of autumn pigmentation and ascribed the autumn colors to carotin, α xanthophyll (showing a four banded spectrum (see Schunck)), β xanthophyll (a water soluble pigment with no spectroscopic properties), a little phyllofuscin and a small amount of another yellow pigment also derived from chlorophyll, which he does not name. One must agree with Tswett (1908b), however, that Kohl's methods are open to serious objection, in that the preliminary boiling of the leaves in water, before the extraction of the pigments with hot alcoholic potash undoubtedly brought about serious decompositions because of the high acidity of the cell sap in autumn leaves. Nevertheless, Kohl's observations do indicate that carotinoids may be expected to decline noticeably during the autumn color change, thus confirming the belief expressed by Tschirch (1884). Kohl states that it is sometimes difficult to demonstrate the presence of carotin (carotinoids) at all in autumn leaves, and concludes that the intense yellow color of some autumn leaves is due to the formation of other yellow pigments.

Tswett's (1908b) study of the pigments of yellow autumn foliage appears to be the most reliable which we have available at present on which to base a definite knowledge of the autumn yellow colors. It is true that Miss Goerrig's (1917) more recent study gave quite opposing results in some particulars, but the apparent contradictions are not wholly irreconcilable when one takes into account the fact that Tswett's and Miss Goerrig's studies differ in at least two very significant points, namely, (1) Tswett studied the pigments in fresh

leaves, while Miss Goerrig first dried the leaves in the air at 40° C. (protected of course from the light), and then ground them to a powder, and (2) Tswett submitted his pigments to confirmatory tests for the various carotinoids (unfortunately omitting, however, a chromatographic analysis), while Miss Goerrig drew her conclusions solely from a Kraus separation between alcohol and petroleum ether. The bearing of these differences in procedure on the conclusions of the two investigators, respectively, will be pointed out below.

Referring first to Tswett's investigation one finds that he plucked the autumn yellow leaves of 19 different plants during the necrobiotic period, and macerated them thoroughly with glass powder, or emery, and MgO (to insure the absence of acids in the extracts) and then extracted the pulp with petroleum ether. This was followed by an extraction with alcoholic petroleum ether. The latter was freed from alcohol by washing with water and the two extracts examined separately both spectroscopically as well as with respect to their adsorption by $CaCO_3$, and also as to their separation in the Kraus procedure, using 80 per cent alcohol and petroleum ether. The first extraction with petroleum ether alone should extract the carotin, if present, and the second extraction with alcoholic petroleum ether should remove the xanthophylls. In addition, extractions were made of the macerated leaves with alcohol alone, and these tested in the Kraus system. The plants examined by Tswett may be found by referring to Tables 1 and 2.

The result of this study was to show that, while traces of chlorophyllins and normal carotinoids were present, the bulk of the pigment of the yellow autumn leaves examined before the postmortal period was a carotinoid-like pigment (or group of pigments) which was almost completely adsorbed from petroleum ether by $CaCO_3$, like the xanthophylls. At the same time the pigment was epiphasic like carotin, i.e., found in the petroleum ether layer in the Kraus separation, in all cases except the extracts[6] from the honey locust (*Gleditsia triacanthos*) and the buckeye (*Aesculus Hippocastanum*). Spectroscopically the pigment showed three absorption bands behind F, but as their position was somewhat variable no measurements were made. Tswett called the pigments (he believed a mixture to be present) autumn xanthophylls. Saponification did not alter their carotin-like property of remaining for the most part in the petroleum ether layer in the

[6] The hypophasic portions of these extracts were unfortunately not examined further by Tswett.

Kraus separation. In the author's opinion the pigments might better have been called autumn carotins, for the behavior of the carotinoids in the Kraus separation unquestionably depends primarily upon chemical composition, as Tswett (1911b) himself has pointed out, while their relation to adsorbents is largely a colloidal phenomenon, as already explained, and is not necessarily related to chemical composition.

Tswett also made a careful study of the question of the alleged (Sorby, 1871a, b, Kraus, 1872, Kohl, 1902) presence of water-soluble yellow pigments in yellow autumn foliage, with most convincing results. He found that hot water decoctions of autumn leaves, obtained with the exclusions of as much air as possible, were scarcely colored at all, and that similar decoctions with dilute acetic acid were pale yellow, while extractions with alkaline water were golden yellow to brown or reddish brown. The more golden colors were destroyed by acid, but the deeper ones persisted. Decoctions with water slightly alkaline with carbonates were likewise golden yellow, and the extracts acted towards acids, alkalies and air like the extracts with distilled water. Tswett obtained further proof of the presence of colorless water- and alcohol-soluble chromogens [7] in autumn leaves (he regards the chromogens as present in green leaves also), which give golden yellow salts with alkalies and oxidize to a dark brown color, by shaking the diluted alcoholic extract of the yellow tulip and maple leaves with chloroform. This removed the color, leaving a colorless hydro-alcoholic layer which acted towards alkalies like the distilled water decoctions from the leaves.

Tswett holds, apparently rightly, that colored salts of the above mentioned chromogens may at times play a part in the coloration of autumn leaves during the necrobiotic period. It would appear that the definiteness of the relation between this period and the true post-mortal period of the leaf is the important factor in determining this type of coloration for Tswett believes that the brown oxidation products of the yellow alkali salts of the colorless chromogen of the leaves play the chief rôle in the postmortal coloration of leaves, a reaction no doubt accelerated by oxidizing enzymes.

Miss Goerrig's (1917) recent study of yellow autumn pigments was an attempt to determine quantitatively the relation of the carotinoids in the green and autumn leaf, just before and during the necrobiotic

[7] These substances are probably flavones which are characterized especially by their yellow color reaction with alkalies.

phases, determining colorimetrically carotin and xanthophylls (as a group) by the Willstätter and Stoll (1913c) method, using potassium dichromate as standard. The study was necessarily most exacting and laborious. In general, the data show that the amount of autumn carotinoids in comparison with the carotinoids in the green leaves just before the necrobiosis varies with the kind of plant and the character of the weather during the latter period, sometimes being more and sometimes less, but that the autumn carotinoids, even when higher than the prenecrosis pigments, never equal quantitatively those present in the leaf in midsummer. In all cases the xanthophylls exceed the carotin. The reader is referred to the original paper for other details. The plants examined are noted in Tables 1 and 2.

From a qualitative standpoint Miss Goerrig's results differ significantly from Tswett's in two particulars, (1) the former denies the existence in autumn yellow leaves of carotinoids differing from the normal plastid carotinoids, (2) and finds water-soluble pigments in abundance so that, "When one compares the color of the extracted meal and the wash waters with the meal before extraction a good idea is obtained of the frequently small significance of carotin and xanthophylls in the autumn leaf pigmentation."

With regard to the first difference, Miss Goerrig's conclusion is certainly open to criticism in that she did not submit her carotin and xanthophyll fractions, obtained by separation between alcohol and petroleum ether, to any confirmatory tests whatsoever. That Miss Goerrig's carotinoids from autumn leaves were probably the same as the mixture which Tswett calls autumn xanthophylls is indicated by the very significant statement that repeated extractions of the petroleum ether solutions with high percentage methyl alcohol were frequently required to separate the xanthophylls from the carotin. It is not difficult to conjecture that such xanthophylls, like Tswett's autumn pigments, would be mostly epiphasic between petroleum ether and *80 per cent alcohol*, the normal xanthophyll solvent in the Kraus separation.

The difference between Miss Goerrig's and Tswett's results respecting water-soluble pigments can not be explained so readily, inasmuch as the former proved that her leaf preparations from both green and autumn leaves did yield strongly colored extracts to both distilled water and to tap water, as well as to very dilute acetone. Leaf powders from leaves dried at 40° C. were used for these tests, while Tswett examined only the fresh tissues. This difference alone may be suf-

ficient to account for the divergence of the results on this point. This possibility should be investigated.

Carotinoids in Autumn and Winter Reddening

The winter reddening of leaves of the English ivy (*Hedera helix*), privet (*Ligustrum vulgare*) and other evergreens, and also that of certain herbaceous plants like *Saxifraga umbrosa*, which retain their leaves in winter, as well as the autumn reddening of the *Rosaceae* and many other individuals of various plant orders is due to anthocyanin formation. The chemistry of autumn and winter reddening, therefore, does not seem to fall within the scope of this monograph. However, from the investigations of Molisch (1902), Tswett (1911b), and Monteverde and Lubimenko (1913b), carotinoids with an anthocyanin-like color are responsible in some cases for autumn and winter reddening.

Schimper (1885) first examined the red pigment in winter foliage of various firs (*Abies*) and other conifers, such as *Thuja ericoides*, *Thuja standishi*, and the common box tree (*Buxus sempervirens*) and found it soluble in alcohol, benzene and CS_2, and that it was extracted from alcohol by the last named solvent. He also noticed the red pigment in various parts of the plant of many varieties of aloës, e.g., *Aloë verrucosa.* Courchet (1888) later investigated the properties of this red pigment and concluded that it was different from carotin and other pigments because it did not give the blue color reaction with con. H_2SO_4. Molisch (1902) believed that he was dealing with the same pigment when he found that the red plastids in winter-red foliage leaves of many (26) varieties of aloës responded, in part at least, to his alkali crystallization method. The pigment crystallized in the form of needles, platelets, prisms or little stars, with a garnet red or yellowish brown color, and gave the usual reaction with con. H_2SO_4, HNO_3, etc. Similar crystals were obtained even more abundantly in the chromoplastids of several varieties of *Selaginella.* Molisch concluded that the winter reddening of aloës and *Selaginella* is due in part to a red carotin (carotinoid).[8]

Tswett (1911b) likewise has found a red carotinoid, undoubtedly the same pigment, in the winter foliage of several plants. These are enumerated in Table 3. He proposes the name thujorhordin for the

[8] Molisch found a similar, if not the same pigment, normally imbedded in a colorless stroma in the common horsetail (*Equisetum arvense*), the pigment itself having been observed first by Schimper (1885).

pigment because he first isolated it from arbor vitæ (*Thuja orientalis*). The pigment is described by Tswett as being ruby red in carbon disulfide, rose in alcohol and yellow in petroleum ether. It is not adsorbed to any extent by $CaCO_3$ from petroleum ether or CS_2 solution, but is almost wholly hypophasic in the separation between petroleum ether and 80 per cent alcohol. Tswett's pigment showed three absorption bands in petroleum ether and four in carbon disulfide.

Spectroscopically the pigment differs from any previously known carotinoid, but Tswett's classification of the pigment as a carotin is subject to the same criticism as his classification of the autumn yellow pigments as xanthophylls. It has already been pointed out that chemical composition, not color and colloidal and other physical and physico-chemical properties, must be the correct basis for the classification of carotinoids. The hypophasic relations of thujorhordin in the Kraus separation would classify it as a xanthophyll. Monteverde and Lubimenko (1913b) and Lubimenko (1914a, and b) have, in fact, already classified it thus under the name rhodoxanthin, which appears to have been the name first applied to this pigment by Tswett (1910b). The discovery of the same pigment in the pond-weed (*Potamogeton natans*) by Monteverde and Lubimenko has been mentioned in an earlier paragraph, and its characteristic properties given.

Monteverde and Lubimenko's study of the *Thuja* pigment differs from Tswett's in that the pigment was isolated in nearly pure crystalline form, making it possible to show that Tswett's four banded spectrum for rhodoxanthin in carbon disulfide was probably due to some admixed carotin or xanthophyll, the pure pigment showing only three bands. According to Monteverde and Lubimenko many of the conifers owe their winter reddening to rhodoxanthin.

Lycopin, the red isomer of carotin, is also involved in winter reddening, according to Lubimenko (1914a). Two conifers whose cone scales owe their winter color to this pigment are mentioned in Table 3. The plants were studied under tropical conditions.

Carotinoids in Flowers

Yellow, orange and orange-red tints are especially abundant among flowers. Marquart (1835) was the first to name the yellow flower pigments, calling them anthoxanthins to distinguish them from the blue, violet and red pigments which he called anthocyanins. Marquart noticed that certain of the anthoxanthins gave a blue color with con.

H_2SO_4, so that observations regarding flower carotinoids may rightfully be said to have begun with this investigator.

Beginning with Frémy and Cloëz (1854), however, it has been recognized that yellow flower colors may be divided, at least roughly, into two groups, one insoluble in water and the other soluble in water. Frémy and Cloëz called the former xanthin, and regarded the pigment of the sunflower (*Helianthus annus*) as the type. The pigments of the water-soluble group were called xantheïn, and the yellow pigment which may be obtained from certain dahlias was regarded as the type. Frémy and Cloëz's xanthin was, of course, a mixture of carotinoids, and their xantheïns are recognized today as anthocyanins and flavones, only a few of which, however, have been isolated and closely studied. We are concerned in this monograph only with the carotinoids, but the subject of yellow flower pigments is somewhat complicated because of the fact that yellow, orange and orange-red pigments of a constitution entirely foreign to that of the carotinoids are frequently the cause of the color of flowers and sometimes associated with the carotinoids in causing the coloration.

It does not appear to be possible to determine with certainty by mere inspection whether a yellow colored flower owes its color to carotinoids or to pigments of the water-soluble group, although Bidgood (1905) states that in general all floral colors of a primrose or sulfur-yellow color are produced by the latter pigments, and that such flowers have a more delicate, transparent appearance. Microscopic observation, however, readily reveals the character of the pigment present, for the carotinoids appear to be always present in flowers in the form of chromoplastids, while the anthocyanins and flavones are always present in solution in the cell sap. There may be some exceptions to both statements but the differentiation is sufficiently general to serve as the basis for determining the character of the flower coloration. Confirmatory tests for carotinoids can, of course, always be carried out. Yellow and orange colored anthocyanins, giving a red color with sulphuric acid, are more common in flowers than are flavones, according to Bidgood, who lists a number of flowers whose color is due to the former, but very few that owe their tints to the latter.

The vast majority of yellow to orange-red colored flowers, however, owe their color to carotinoid containing chromoplastids. Thudichum (1869) gave a list of 32 flowers whose yellow pigment he regarded as due to lutein. Carotinoids have since been demonstrated in practically all these cases. Gregor Kraus (1872) first noticed the spectro-

scopic similarity of alcoholic extracts of yellow flowers with the xanthophyll which he obtained from green leaves by his well-known method of separation of the green and yellow chloroplastid pigments. Sorby (1873) believed that yellow and orange colored flowers were colored by a mixture of the various xanthophylls and xanthines which he described and which have already been reviewed in detail. Dippel (1878) also noticed the similarity between the absorption spectra of flower extracts (he used the golden-yellow flowers of the California poppy (*Eschscholtzia californica*) and the yellow pigments of chloroplastids. Hansen (1884c) apparently obtained the first crystals of carotinoid from yellow flowers, but their probable impurity is indicated by their ease of solubility in both alcohol and petroleum ether, crystalline carotin being practically insoluble in the former and crystalline xanthophylls in the latter solvent. Hansen called the flower plastid pigment lipochrome, because of its similarity in properties to the animal lipochromes which were being studied by Krukenberg (1880-1886) about that time.

Schimper (1883, 1885) first observed that the orange-yellow pigments in the chromoplastids of certain flowers existed naturally in crystalline condition, while in others the pigment was granular or amorphous. These observations were greatly extended by Courchet (1888), who used the name chromoleucites first proposed by van Tieghem in place of chromoplastids. The former has not had such general use as the latter. Courchet's extensive investigation was not confined to the yellow and orange pigments of flowers and fruits, but covered the anthocyanins as well. He described very minutely the morphology and organization of the chromoplastids and the pigments contained therein for many flowers and fruits. He found a very interesting relation to exist between the color of a pigment and the form which it assumes in the plant. He showed clearly how to distinguish between red anthocyanin pigments dissolved in the cell sap and red plastid pigments frequently deposited in crystals in the plastids, the latter being characterized by their blue color reaction with con. H_2SO_4; in form and color and reactions with reagents they were shown to be identical with the red crystals in tomato plastids, which were believed at that time to be identical with the carotin of carrots. He demonstrated that yellow-orange and red-orange colors in flowers are always found in the chromoplastids, frequently in the form of crystals whose rhombohedral platelets or prisms were recognized as extraordinarily closely related to the carotin of carrots and green leaves. He

pointed out distinctly the difference between yellow flower pigments dissolved in the cell sap and yellow flower pigments deposited, apparently always, in amorphous condition in the plastids. Courchet was successful in recrystallizing the red, red-orange and yellow-orange plastid pigments, but not the yellow plastid coloring matters. The latter, however, gave the same color reactions with the lipochrome reagents as the crystallizable pigments, and their great solubility in absolute alcohol shows clearly that they are to be classified as xanthophylls. It is not so certain that the crystalline forms were carotin in all cases on account of their somewhat variable solubility in alcohol.

Following Courchet, carotin crystals were obtained by Immendorff (1889) from extracts of the flowers of various members of the buttercup (*Ranunculus*) family and from various members of the hawkbit (*Leontedon*) family, probably especially the so-called autumn dandelion (*Leontedon autumnale*). Möbius (1885) had already shown that the peculiar oily appearance of the yellow buttercups, which is the cause of their common name, is caused by the pigment being dissolved in the cells in oil and not present, as usual, in plastid form. This flower therefore appears to present an exception to the general rule that flower carotinoids are present in chromoplastids. In this connection, the idea of Hilger (1894) and his pupils Wirth (1891) and Kirchner (1892), that the carotin pigment of the pot marigold flower (*Calendula officinalis*) is a mixture of sterol esters of various fatty acids and an unnamed colorless hydrocarbon, has been completely disproved. Pabst (1892) proposed the same idea to explain the constitution of the pigment of the red pepper (*Capsicum annum*) and Ehring (1896) the pigment of the tomato (*Lycopersicum esculentum*). Even if these authors had regarded carotin as a sterol-like substance in union with fatty acids, which does not appear to have been the case, the hydrocarbon character of carotin and the fact that xanthophylls apparently contain no hydroxyl group would render their conclusion untenable.

We are dependent for our present knowledge of the distribution of carotinoids among flowers upon the observations of Tammes (1900), Kohl (1902), Schunck (1903), Tschirch (1904), Bidgood (1905) and van Wisselingh (1915). Tammes, Kohl and van Wisselingh submitted the flowers examined by them to one or more of the microchemical crystallization methods previously described, the last named investigator supplementing these in certain instances with additional tests on the crystals thus formed in order to determine, if possible,

whether the crystals were carotin or xanthophylls. Schunck's combined separation and photospectrographic procedure, together with the effect of certain reagents on the absorption bands, has already been described in detail. Schunck reported especially the distribution of his L. B. and Y. xanthophylls, respectively, in a number of common yellow flowers. The author is of the opinion that Schunck's work can be relied on merely as having shown that xanthophyll carotinoids are present in the particular flowers examined by him. The author recently [9] sought to verify Schunck's observation that the pigment of the common dandelion (*Taraxacum officinale*) is compared solely of B. xanthophyll, which corresponds with Tswett's α' and α'' xanthophylls, with the view of determining the influence of a single xanthophyll on the coloration of egg yolk when fed to laying hens. Only the purest yellow parts of the dandelion head were examined. Applying relative solubility, spectroscopic and chromatographic adsorption methods to the extracted pigment, it was found, however, that carotin and at least three xanthophylls were present, carotin being especially abundant. Xanthophyll β (Schunck's Y. xanthophyll), characterized by the peacock-blue color of its alcoholic solution on treatment with HCl, was among the xanthophylls present.

Tschirch used a still different method for his flower studies. The alcoholic extracts were submitted to the capillary analysis procedure first introduced by Goppelsroeder (1901), and the carotinoid character of the principal yellow zone confirmed spectroscopically.

This capillary method of separation has not been mentioned previously so that a few statements concerning its character may be made at this point. The alcoholic extract of the flower whose pigments are to be examined is placed in a cylindrical vessel with a flat bottom and strips of thick, fat-free paper, such as that used for the Adams' milk-fat analysis, are immersed in the solution to a depth of about one centimeter. The strips used by Tschirch were 5 cm. wide, 18 cm. long and about 1 mm. thick. These strips are hung from a support. During the course of several hours the pigmented extract gradually rises on the paper and as it does so differentiates itself into colored zones, strikingly similar in appearance to those obtained in the Tswett chromatographic analysis. When the capillary rise has ceased the paper strips are removed, dried, and the various colored zones separated with the scissors. The pigment in the individual zones is purified by repeating the capillarity until the paper takes up only one

[9] Unpublished investigation.

pigment. The method is strikingly pretty but has the serious objection that the various carotinoids are likely to lose some of their characteristic properties through oxidation before a pure pigment is obtained. According to Tswett (1906c), the differentiation into zones is not due to adsorption, as in his method, but merely to a combined effect of surface evaporation and precipitation of pigments having varying degrees of insolubility in the increasingly dilute alcohol. It is difficult to believe, however, that colloidal adsorption does not play a part in this phenomenon.

Bidgood's addition to the subject of flower pigments is a list of flowers whose orange, brown and green tones are due to the combined effect of carotinoids and crimson and blue anthocyanins.

The author has attempted to collect in Tables 4, 5, 6 and 7 the results of the work of the various investigators mentioned. It is seen that a very large number of yellow flowers owe their color wholly or in part to carotinoids. It should be understood that the tables include only those which have been studied, and that the lists are not necessarily complete. A few statements may be necessary in explanation of the separate tables. The carotin-containing flowers in Table 4 must not be regarded as containing this pigment only. As a matter of fact carotin apparently never exists alone, at least in flowers and leaves, although it may be the predominating pigment, as in the corona of the poet's Narcissus, and the daffodil where it is found already crystallized in the plastids (Courchet, Bidgood, van Wisselingh). The orange colored pigment of the pollen of the so-called mullein (*Verbascum thapsiforma* L.) appears to consist wholly of carotin (Bertrand and Poirault, 1892). The flowers which are starred in Table 4 were placed there largely because they have been found to yield microchemical crystals by the acid method, which may, at least provisionally, be regarded as specific for carotin on account of the great sensitiveness of xanthophylls toward acid. The presence of carotin in the other flowers in Table 4 has been substantiated by other observations. Arnaud determined quantitatively that the flower petals of the sweet violet (*Viola odorata*) contain 124 mg. carotin per 100 gms. of dried petals. The xanthophyll-containing flowers of Table 5, similarly, do not necessarily contain this class of carotinoids only, although it is possible that this may be the case for those which are starred, judging from van Wisselingh's study of this question. This investigator's conclusion that the flowers which are double starred in the table contain only one xanthophyll should be substantiated by

chromatographic analysis. The collection of flowers in Table 6 signifies that we know as yet only that carotinoids are present in these flowers. Whether the color effects are also due in part to yellow hued non-carotinoids is not known. The cases where this fact appears to have been established are reported in Table 7, which includes as well a few of the known cases where other color tones are due in part to carotinoids.

To summarize the whole subject of flower carotinoids very briefly, it is clear that much work remains to be done before our knowledge can be regarded as complete regarding the character and distribution of the individual carotinoids among the yellow flowers.

TABLE 4. YELLOW FLOWERS IN WHICH CAROTINS HAVE BEEN DEMONSTRATED

*_Abutilon Darwinii_ Hook (Flowering Maple), Tammes.
Aloë verrucosa, Molisch.
Asclepias Curassavica L. (Milk weed), van Wisselingh.
Asphodelus cerasifer L. (Asphodel), Courchet.
*_Calceolaria rugosa_ Hook (Lady-slippers), van Wisselingh.
*_Chelidonium majus_ L. (Celandine Poppy), Tammes.
Isatis Tinctoria L. (Dyer's Woad), van Wisselingh.
Liriodendron tulipifera (Tulip tree), Schrötter-Kristelli.
*_Manettia bicolor_ Paxt., Tammes.
Momordica balsamina (Balsam Apple), G. and F. Tobler.
Narcissus poeticus L. (Poet's Narcissus), Courchet, Bidgood, van Wisselingh.
Narcissus Pseudo-narcissus L. (Daffodil), van Wisselingh.
*_Nonnea lutea_ D. C., Tammes.
*_Primula officinalis_ (Cowslip), Tammes.
*_Siphocampylos bicolor_ G. Dow, Tammes.
*_Stilophorum diphyllum_ Nutt., Tammes.
Taraxacum officinale Weber (Common Dandelion), Palmer.
*_Trollius asiaticus_ L. (Globe Flower), Tammes.

* Carotin by the acid microchemical crystallization method.

TABLE 5. YELLOW FLOWERS IN WHICH XANTHOPHYLLS HAVE BEEN DEMONSTRATED

Calceolaria (Lady-slippers), Schunck.
**_Calendula arvensis_ L., van Wisselingh.
Calendula officinale L. (Pot Marigold), Schunck.
Cheiranthus cheira L. (Wall-flower), Schunck.
**_Chelidonium majus_ L. (Celandine Poppy), van Wisselingh.
Chrysanthemum (probably _frutescens_ L.) (Marguerite), Schunck.
Cytisus Laburnum L. (Broom Flower), Schunck.
*_Dendrobium thyrsiflorum_ Rchb. fil. (Orchid), van Wisselingh.
**_Doronicum Pardalianches_ L. (Leopard's Bane), Schunck, van Wisselingh.
*_Gazania splendens_ Hort., Kohl, van Wisselingh.
Helianthus annus (Sunflower), Schunck.
**_Hieraceum aurantiacum_ L. (Orange Hawkweed, or Devil's Bit), van Wisselingh.
Isatis tinctoria L. (Dyer's Woad), van Wisselingh.
**_Lilium croceum_ Chaix., van Wisselingh.
Mimulus moschatus L. (Musk plant), Schunck.

* Flowers containing xanthophylls and no carotin, according to van Wisselingh.
** Flowers whose pigmentation is due to one xanthophyll only, according to van Wisselingh.

Narcissus Pseudo-narcissus L. (Daffodil), Schunck, van Wisselingh.
Primula officinalis (Cowslip), Kohl.
Ranunculus acris L. (Buttercup), Kohl, Schunck.
Raphanus raphanistrum L. (White Charlock), Schunck.
Ribes aureum (Golden Current), Kohl.
***Spartium junceum* L. (Spanish Broom), van Wisselingh.
Tagetes erecta (Marigold), Schunck.
Taraxacum officinale Weber (Common Dandelion), Schunck, Palmer.
Tropaeolum majus (Nasturtium), Schunck.
Tulipa Gesneriana L. (Common garden Tulip), van Wisselingh.
Tussilago Farfara L. (Coltsfoot), Schunck.
Ulex europoeus (Gorse), Schunck.
Verbascum species (Mullein), Kohl.
Viola tricolor L. (Pansy), Schunck.

** Flowers whose pigmentation is due to one xanthophyll only, according to van Wisselingh.

TABLE 6. YELLOW FLOWERS IN WHICH CAROTINOIDS HAVE BEEN DEMONSTRATED

Abutilon megopotamicum (Flowering Maple), Tammes.
Abutilon nervosum (Flowering Maple), Kohl.
Adonis vernalis (Spring Adonis), Tammes.
Alyssum saxatile (Golden-tuft), Tammes.
Aster species, Courchet.
Buphthalmum salicifolium, Tschirch.
Cacalia coccinea, Tschirch.
Caltha palustris (Marsh Marigold), Kohl, Tammes, Tschirch.
Chrysanthemum frutescens (Marguerite), van Wisselingh.
Clivia miniata Regel, van Wisselingh.
Colutea media (Bladder Senna), Tschirch.
Corydalis lutea D. C. (Lark's Spur), van Wisselingh.
Cucurbita foetissima (Calabazilla), Kohl.
Cucurbita melanosperma A. Br., van Wisselingh.
Cytisus Laburnum L. (Broom), van Wisselingh.
C[illegible] Koch. (Broom), van Wisselingh.
D[illegible] Tenore (Leopard's Bane), Kohl, Tammes, Tschirch.
Doronicum plantagineum L. *excelsum,* van Wisselingh.
Doronicum Pardalianches, Tschirch.
Epimedium macrantheum, Tammes.
Eranthis hyemalis Salisb. (Winter Aconite), Tammes, van Wisselingh.
Erysimum Perofskianum Fisch. and Mey., van Wisselingh.
Ferula species (Giant Fennel), van Wisselingh.
Forsythia Fortunéi (Golden Bell), Tammes.
Forsythia viridissima (Golden Bell), Tammes, Kohl, Tschirch.
Fritillaria Imperialis (Crown Imperial), Tammes, Kohl, van Wisselingh.
Gaillardia splendens, Tschirch.
Gazania species, Tschirch.
Genista racemosa, Tammes.
Genista tinctoria (Dyer's Greenwood), Courchet.
Geum montanum, Tschirch.
Gongora galeata Reichb., van Wisselingh.
Helenium autumnale (Sneezewood), Tammes.
Hemerocallis Middendirffii Trautv. and Mey (Yellow Day Lily), van Wisselingh.
Hieracium murorum L. (Hawkweed), van Wisselingh.
Hieracium Pilosella (Mouse-ear Hawkweed), Courchet.
Impatiens Noli-tangere (Touch-me-not), Kohl, Tammes.
Inula Helenium L. (Elecampane), van Wisselingh.
Iris Pseudacorus L., van Wisselingh.

Kerria japonica D. C. (Japanese Rose), Kohl, Tammes, Tschirch, van Wisselingh.
Kleinia Galpini (Groundsel), van Wisselingh.
Kniphofia alooides (Torch Lily or Poker Plant), Tammes.
Ladamun hybridum, Kohl.
Leontedon autumnalis (Autumn Dandelion), Immendorff.
Leontedon Taraxicum (probably Common Dandelion), Tschirch.
Lilium bulbiferum, Hansen.
Loasa lateritia, Kohl.
Lycaste aromatica, Tammes.
Meconopsis cambria Vig. (Welsh Poppy), van Wisselingh.
Melilotus officinalis (Sweet Clover), Tschirch.
Nuphar luteum Sibth. (European Pond Lily), van Wisselingh.
Oenothera biennis (European Evening Primrose), Tammes, Kohl.
Physalis Franchetti (Chinese Lantern Plant), Tammes.
Ranunculus auricomus (Buttercup), Tammes, Kohl.
Ranunculus Ficaria, Tammes, Kohl.
Ranunculus Gramineus, Tammes.
Ranunculus repens, Kohl, Tammes.
Rosa species, yellow flowers, Hansen, Kohl.
Rudbeckia Newmanii (Cone Flower), Tammes.
Silphium perfoliatum (Cup plant), Kohl.
Sinapis alba L., van Wisselingh.
Sisymbrium Sophia, Tammes.
[illegible] (Bird-of-Paradise flower), Tammes, Kohl.
Tagetes patula (Marigold), Courchet, Kohl, Tammes.
Telekia speciosissima, Tschirch.
Thermopsis lanceolata R. Br., van Wisselingh.
Tillandsia splendens, Tammes.
Tritonia aurea (Blazing Star), Kohl, Tschirch.
Trollius europaeus (Globe Flower), Tammes, Kohl.
Tropaeolum majus (Nasturtium), Courchet, Tammes, Kohl.
Tropaeolum minus (Nasturtium), Kohl, A. Meyer.
Tulipa Gesneriana L., van Wisselingh.
Tulipa hortensis Gaertn., van Wisselingh.
Uvalaria grandiflora (Bellwort), Tammes.
Verbascum Thapsiforme (Mullein), Tschirch.
Viola odorata (Sweet Violet), Molisch, Kohl.
Viola biflora, Tschirch.
Viola cornuta L. var. Daldowie yellow (Horned Violet), van Wisselingh.
Viola lutea (Yellow petal Violet), Tschirch, Tammes.
Waldsteinia geoides, Tammes.

TABLE 7. FLOWERS CONTAINING CAROTINOIDS AND OTHER PIGMENTS

Allium siculum (Onion), Courchet. Chiefly carotinoids with some chlorophyll, giving brown color.
Armeria vulgaris (Thrift), Courchet. Chiefly carotinoids, some soluble yellow non-carotinoids.
Atropa belladona (Belladonna), Courchet. Chiefly carotinoids, some soluble non-carotinoids.
Bulbine semibarbata, Courchet. Chiefly carotinoids, some soluble yellow anthocyanins.
Crocus sativus, Tschirch. Some carotinoids in flower petals, also Safran. Pollen grains Safran.
Cypripedium Boxollii, C. insigne, C. argus. (Lady's Slipper), Bidgood. Chlorophylls back of chromoplastids giving brown effect.
Eschscholtzia californica (California Poppy), Courchet. Chiefly anthocyanins, with some xanthophylls (?).
Geum coccineum, Courchet. Partly orange colored carotinoids and partly orange colored anthocyanin.

Maslevallia Veitchii, Bidgood. Purple cell sap and carotinoid chromoplastids.
Narcissus Tazetta (Polyanthus Narcissus), Bidgood. Chiefly yellow anthocyanin with some carotin (?).
Odintiglossums (Orchids), *Oncidiums* (Orchids), *Tropocolums* (Nasturtiums), Bidgood. Many varieties of these have crimson anthocyanin in epidermal cells and yellow carotinoids along inner walls of same cells.
Wallflowers, Bidgood. Some have crimson sap and carotinoids in plastids.
Yellow tulips, Bidgood. Blue anthocyanin in epidermal cells, overlying yellow chromoplastids in staminal filaments, giving green effects.

Carotinoids in Fruits

A large number of yellow, orange and red colored fruits have been examined by various investigators for the nature of the pigment. The coloring principles found in most cases can be classified with the carotinoids. For the majority of the fruits, however, we have the observation of only one investigator and this in many cases has been very inadequate for the purpose of properly classifying the kind of carotinoid present. Even for many of the fruits that have been studied by more than one investigator we only know, as yet, that carotinoids are the cause of the pigmentation. There is great need for an application of the Tswett system of analysis to the pigments of these fruits. Only in one case, namely, the tomato, has a thorough study of the pigment been made. This work will be referred to in detail presently.

The fruits for which only a single observation has been made will be reviewed first, briefly, considering the cases chronologically. The various fruits for which more than one observation is available will be considered separately.

Thudichum (1869) classified the pigment of the fruit of *Crataegus crus-galli* (Cockspur thorn) and *Cyphomandra betacea* (Tree tomato) as luteins. It seems reasonable to suspect that carotinoids are involved, but nothing further is known regarding their character.

Gregor Kraus (1872) observed orange-red, round or spindle forms in the fruit flesh of *Solanum pseudo-capsicum* (Jerusalem Cherry), but the pigment involved, which is obviously a carotinoid, is not known further.

Schimper (1885) observed amorphous red and orange-yellow pigment forms in the fruit of *Bryonia dioica* (Bryony) and red amorphous pigment in the fruit of *Loniceria tataria* (Honeysuckle), but these pigments, apparently carotinoid in nature, have not been examined in detail.

Courchet (1888) not only examined the character of the pigment in the plastids of several fruits but extracted the pigment and recrys-

tallized it. For example, carmine colored rhombohedral crystals with a few orange-red trapezoidal forms were obtained from the ether extract of the fruit flesh of *Cucumis melo* (Muskmelon). In the case of the little tomato-like fruits of *Eugenia uniflora* (Pitanga or Surinam cherry) a red anthocyanin was found in the epidermis cells, and orange colored chromoplastids in the pericarp which crystallized in the form of red-orange rhombic plates, and is thus obviously carotinoid in nature. The ether extract of the berries of *Douce-amere* and *Solanum corymbosum*, however, deposited both a yellow amorphous xanthophyll-like pigment and thin, pale, red, rhombohedral platelets, frequently grouped together in clusters. The latter may be carotin.

Desmouliere (1902) extracted a yellow pigment from the juice of *Prunus armeniaca* (Apricot) with amyl alcohol and the residue from this solution gave the lipochrome reaction with con. H_2SO_4. He concluded that the pigment was probably carotin but there is no evidence that only one carotinoid was present. The character of the pigment of the apricot and also that of peaches should be examined in the light of our present knowledge of the carotinoids.

Kohl (1902) obtained carotinoid crystals by the Molisch microchemical crystallization method in the case of the fruits of *Berberis vulgaris* (Common Barberry), and several kinds of *Clivias* and *Cotoneasters*, but his conclusion that carotin only was involved we know now to be a mistake.

Tschirch (1904) made a capillary colorimetric analysis of the alcoholic extract of the little fruits of *Euonymous europaeus* (European Spindle-tree) and obtained several yellow to red-orange zones. The chief zone of the latter color unquestionably showed the spectrum of carotin. Whether other carotinoids are present is not known.

Duggar (1913) obtained spectroscopic and physiological evidence that the principal pigment of the carpellary tissue of *Momordica charantia* (Balsam Pear) is carotin but that lycopin, the red tomato pigment, characterizes the bright red aril of the seed.

Monteverde and Lubimenko (1913b) found the bright red pulp of the tropical fruit *Trichosanthus* to owe its pigment to lycopin and carotin, chiefly the former.

Lubimenko (1914a) examined the pigment of a large number of fruits in the famous botanical gardens at Buitenzorg, Java, in order to determine the effects of the tropical conditions on the development and character of the pigment. The predominating pigment found was lycopin or a closely related pigment which Lubimenko calls lycopin-

oid, because of slight differences in the relative intensity of the first two absorption bands as compared with the same bands of lycopin, and because of a greater ease of solubility in absolute alcohol and glacial acetic acid. In the case of the fruit of *Arum orientale,* the chief pigments were carotin and xanthophylls, true lycopin making up only a small part of the pigment. Various *Aglaonema* fruits, such as *Ag. nitidum* Kunth, *Ag. oblongifolium* Kunth, *Ag. oblong.* variety *Curtisii,* and *Ag. simplex Bl.* were found to contain variable amounts of yellow pigments besides lycopin. The following fruits appeared to contain chiefly lycopin: *Actinophlocus angustifolius* Becc., *Actinophlocus macarthurii* Becc., *Archonthophoenix Alexandrae* H. Wendl., *Calyptrocalix spicatus* Blume, *Erythroxylum nova-granadense, Nenga Schefferiana* Becc., *Nertera depressa* Banks and Soland, *Pandanus polycephalus* Lam., *Ptychandra glauca* Scheff., *Ptychosperma elegans* Blume, *Sinaspadix Petrichiana* Hort., *Solanum decasepalum, Tanernomontana pentastycha Scheff.* Especially interesting were the fruits of *Gonocarium obovatum* Hocr. and *Gon. pyriforme* Scheff., the bands of whose pigment in carbon disulfide solution were intermediate between the characteristic bands of carotin and lycopin. Lubimenko regarded the lycopin of the fruit of the palm *Areca Alicae* W. Hill as a lycopinoid because its first two spectroscopic absorption bands in carbon disulfide were of equal intensity while the second band in the case of lycopin is more intense than the first.

Van Wisselingh (1915) obtained a positive carotinoid reaction on the fruits of *Viburnum Opulus* (European cranberry bush), using the Molisch microchemical crystallization method, but made no further study of the crystals.

Gill (1918) has found carotinoids in palm oil, the commercial product which is obtained from the fruits of certain *Palmaceæ,* particularly *Eloeis guineesis* L. (Jacq.), which form vast forests along the West Coast of Africa, and *Eloeis melanococca,* Gärb. (*Alfonsia oleifera,* Humb.) which is cultivated in the West Indies and in South America. Gill's observations are of interest in the light of Lubimenko's study of the pigments of the palm fruits, mentioned above.

Asparagus berries. Thudichum (1869) classified the pigment with the luteins. Hartsen (1873b) described the red granules of pigment in the berries and stated that red-colored crystalline tablets of the pigment were insoluble in water, soluble in alcohol and ether and especially so in petroleum ether. The pigment thus appears to be

carotin, but whether other carotinoids are present has not been determined.

Ampelopsis hederaceæ. Hansen (1884) classified the pigment of the fruit as a lipochrome and Kohl (1902) obtained carotinoid crystals by the Molisch microchemical method.

Aglaonema commutatum Shott. Tammes (1900) obtained positive carotinoid color tests on the chromoplastids of the fruit. Van Wisselingh (1915) made a detailed study of the microchemical crystals obtained by the Molisch method and concluded that the chief pigment is lycopin, but that other carotinoids are present also.

Citrus limonum (Lemon). Neither Kohl (1902) nor Tschirch (1904) could find evidence of carotinoids being involved in any way in the pigmentation of the yellow skin of this fruit.

Cucumis citrullis (Watermelon). A. and G. de Negri (1879) first isolated the pigment of the flesh of this fruit and called it rubidin because of its red color. Red, needle shaped crystals were described, soluble in ether, benzine and chloroform, forming yellow or yellow-red solutions, and in carbon disulfide forming a magnificent rose-colored solution. The insolubility of the crystals in alcohols and the characteristic three-banded absorption spectrum which was found to be identical with that of the red tomato pigment obviously classifies the pigment as a carotin if not as lycopin itself, as there is good reason to believe. Courchet (1888) also crystallized the watermelon pigment and found that the crystals resembled completely in form and in color those obtained from the tomato. Monteverde and Lubimenko (1913b) have definitely confirmed its identity with lycopin, as well as to show that carotin and xanthophyll are also present in the red fruit pulp.

Cucurbito pepo L. (Pumpkin). Arnaud (1885) stated that he obtained crystals of pigment from the flesh of this fruit which were identical in properties with carrot carotin. Schrötter-Kristelli (1895b) later made a closer study of the pigment of this family of plants, using for the source of his material the thin deep-red outer layer of the pericarp of so-called Turk's-cap gourd. The pigment was not found to be readily extractable by alcohol, even by hot absolute alcohol, but was readily soluble in petroleum ether, ether, chloroform and carbon disulfide. The recrystallized pigment was found to be identical in solubility and in its reactions with carotin, especially the emerald green color on addition of HCl to the alcoholic solution of the pigment. The conclusion seems justified that carotin is the chief

pigment of the fruits of the gourd family, including pumpkins and the various yellow fleshed squash varieties. Whether other carotinoids are also present has not been determined.

Gill (1918) has recently stated that carotin is found in yellow squash, the statement being based on the use of the Crampton-Simons palm oil test which this author has found to be due to carotin (probably carotinoids in general).

Momordica balsamina (Balsam Apple). G. and F. Tobler (1910) first studied the pigment of this fruit and believed that two pigments were present; a yellow one soluble in alcohol, ether, benzene and fatty oils, the ether solution showing the absorption bands, 478-465μμ and 435-415μμ; a ruby red pigment, extractable by cold alcohol, but not by benzene, but soluble in both of these solvents as well as in ether, chloroform and fatty oils, which failed to show the lipochrome reactions with H_2SO_4 and I_2-KI, but which showed the following four-banded spectrum in benzene.

I. 513-496μμ; II. 487-446μμ; III. 455-443μμ; IV. 437-425μμ.

The yellow pigment was found chiefly in the exo- and mesocarp. Its solubilities and spectra indicate that it is a xanthophyll. The red pigment was found chiefly in the endocarp. Its absorption spectrum in alcohol resembles closely that of lycopin but the other properties are at variance. Duggar (1913) also examined the balsam apple pigment and found the carpellary tissues to be yellow to orange as did the Toblers, and the aril to have a bright red color. Duggar regards the latter pigment to be lycopin, on spectroscopic grounds, but the failure of the pigment to show other characteristic carotinoid properties, as found by the Toblers, remains to be explained.

Physalis alkekenzi (Strawberry Tomato, Winter or Bladder Cherry). Thudichum (1869) classified the pigment of this fruit as a lutein, and Tammes (1900) obtained positive carotinoid color reactions with the plastid pigment. Monteverde and Lubimenko (1913b) regard the pigment as carotin, but differing from it by the comparative intensity of the absorption bands. They call it carotin B.

Arum italicum (Wild Ginger). Schimper (1885) observed red amorphous pigment in the plastids of the berries of this plant. Courchet (1888) also observed the brick-red plastids, and obtained red-orange lamella and carmine-red rhomboids from the yellow-orange ether extract. Kohl (1902) secured microchemical carotinoid crystals using the Molisch method. Carotin seems to be one of the pigments

involved here. Whether other carotinoids are present remains to be determined.

Lonicera xylosteum (Honeysuckle). Schimper (1885) stated that he observed red and orange-yellow crystals in the plastids of the fruit. Molisch (1896) and Kohl (1902) obtained microchemical crystals by the alkali method of the former worker. Nothing further is known regarding the carotinoids present.

Physalis franchetti (Chinese Lantern Plant). Carotin is apparently the chief if not the only pigment present in the fruit from the observations of Tammes (1900), Tschirch (1904) and van Wisselingh (1915). Tammes obtained splendid microchemical crystals by the acid method. Tschirch found only one characteristic orange-colored zone in the capillary analysis of the alcoholic extract, showing the carotin bands.

The crystals which van Wisselingh obtained by the alkali microchemical method were insoluble in phenol-glycerine, a property which he found to be characteristic of carotins.

Sorbus aria, Crantz (White Beam-tree). Thudichum (1869) classified the pigment of the fruit among the luteins. Tammes (1900) obtained carotinoid color tests on the plastid pigment. Van Wisselingh (1915) found three types of crystals in the fruit wall after 15 months' treatment by the Molisch microchemical method; (1) thin orange-red platelets, often parallelograms, (2) orange crystal bundles, and (3) orange-yellow crystal masses. The classification of the carotinoids present remains to be made.

Tamus communis (Black Bryony). Both Hartsen (1873) and Courchet (1888) obtained red crystals from extracts of the berries, but did not name the pigment. Van Wisselingh (1915) has made a closer study and obtained microchemical evidence of lycopin and xanthophylls, but not of carotin in the fruit. An analysis of the pigments present using the Tswett (1911a) procedure would be of value in confirming this interesting case of carotinoid distribution.

Rosa species. Both Tammes (1900) and Kohl (1902) demonstrated carotinoids in fruits of this family, the former using both colorimetric and alkali crystallization methods and the latter the microchemical crystallization (alkali) method only on the plastids. The dark-orange capillary zone which Tschirch (1904) examined showed the carotin spectrum, using the fruit skins of *Rosa canina* (Dog Rose), as source of his material. Monteverde and Lubimenko (1913b), however, have isolated lycopin crystals from the dried fruit pulp, but they neverthe-

less regard it as a minor constituent of the pigments of this fruit. The microchemical Molisch crystals which van Wisselingh (1915) obtained from the orange fruits of *Rosa rugosa* Thumb. (Rugosa Rose) dissolved readily in phenol-glycerine, which is indicative of xanthophylls. The pigmentation of the various rose fruits thus appears to vary.

Sorbus aucuparia (European Mountain Ash). Immendorff (1889) believed carotin to be the pigment of the fruit of this plant. Both Tammes and Kohl obtained microchemical crystals by the alkali method, and van Wisselingh (1915) by the acid method as well. The latter investigator made a closer study of the crystals obtained and found red and orange-red platelets insoluble in phenol-glycerine, and orange and yellow-orange platelets and needles which dissolved readily in this reagent. Both carotin and xanthophylls appear to be present, and a Tswett chromatographic analysis of the mixed pigments would probably give the characteristic chloroplastid display of carotinoids.

Citrus aurantium (Orange). Tammes (1900) obtained positive carotinoid color reactions on the skin plastids but failed to secure crystals after a short (18 day) submission to the alkali microchemical crystallization method. Kohl examined the pigment of the pericarp, and found only spectroscopically inert pigment, although he thought there might be traces of carotin (carotinoids) present. Schunck (1903) studied the skin pigment of several varieties of oranges and found considerable amounts of water-soluble (anthocyanin?) pigments, especially in the red skin varieties (Blood orange, Seville orange and Tangerines). He found, however, that the alcoholic extracts yielded crystals of chrysophyll (carotin) and showed spectroscopically the presence of acid derivatives of B. and Y. xanthophylls. Tschirch (1904) also obtained proof of the presence of water-soluble non-carotinoid pigments in the orange skin. His spectroscopic study of the principal carotinoid pigment, secured by the capillary method, did not give satisfactory results. Gill (1918) has obtained a carotin (carotinoid) test with orange skin extracts, using the color reaction mentioned above. A more exact study of the orange pigments, using chromatographic and solubility methods, as well as the improved microchemical methods, would seem desirable.

Solanum dulcamara (Bittersweet). Thudichum (1869) classified the pigment as lutein. Hartsen (1873) obtained red crystals identical with those from *Tamus communis* and Asparagus berries. Schimper (1885) observed red crystals in the fruit plastids, and Tammes (1900)

crystallized them by the Molisch method. According to Lubimenko (1914a) lycopin is the chief pigment present. Van Wisselingh (1915) obtained crystals of pigment by the acid microchemical method as well as by the alkali method, and on further study concluded that lycopin is the chief pigment, but that another orange-red carotinoid is present also, which fails to react towards the I_2-KI reagent. A further study of the latter pigment, which van Wisselingh found in other fruits also, would seem to be desirable. It has previously been considered that the frequent failure of the iodine reaction was characteristic of the animal lipochromes only, and was, in fact, one point of difference between the plant and animal lipochromes. This differentiation seems to break down in the light of van Wisselingh's results.

Capsicum annum (Red Pepper). The red pepper pigment has interested a number of plant biologists. Thudichum (1869) first classed it with the luteins. Pabst (1892) was unable to identify it spectroscopically with carotin. Kohl (1902) regarded the pigment as completely identical with carotin, but in this he was mistaken, for Tschirch (1904) recognized the close relation of the pepper pigment spectrum with that of lycopin. Duggar's (1913) spectroscopic observations led him to conclude that lycopersicin (lycopin) is the pigment of both the skin and flesh of the red pepper. While van Wisselingh (1915) obtained a positive carotinoid test using the alkali crystallization method, he does not classify the Capsicum fruit among those containing lycopin. It should be stated that the measurements of the absorption bands of lycopin, given by Tschirch (1904), do not correspond exactly with the lycopin bands (in the same solvent) given by Willstätter and Escher (1910). Monteverde and Lubimenko (1913b) found the red pepper pigment to be spectroscopically identical with lycopin but because of the ease of solubility of the crude pigment in alcohol, in opposition to the usual difficult solubility of lycopin in this solvent, they have named it lycopin B.

Lycopersicum esculentum (Tomato). The red tomato pigment has been by far the most extensively studied of the fruit pigments of the carotinoid class, and is the only one, in fact, for which we possess at present definite chemical knowledge that it is not identical with the usual carotin and xanthophylls of the chloroplastids.

Millardet (1876), who first investigated the tomato pigment, recognized that it is not identical with the orange and yellow pigments which characterize other fruits. He therefore proposed the name solanorubin for the pigment. It is recognized now that the name was

not very wisely chosen. The name proposed for the pigment by Schunck (1903), who apparently was unfamiliar with Millardet's investigation, namely, lycopin, is more generally used at present, especially since Willstätter and Escher (1910) adopted it in their thorough chemical study of the pigment. Duggar (1913) has offered the name lycopersicin as being more suitable, but in spite of Duggar's very valid arguments against the name lycopin, the latter appears likely to become the prevailing term for the coloring matter.

Millardet not only obtained the tomato pigment in crystalline condition, but also observed the crystals in the flesh of the ripe fruit. The crystalline pigment was described by him as being insoluble in water, soluble in alcohol at higher temperatures, and easily soluble in CS_2, $CHCl_3$ and benzene. It showed a characteristic spectrum in CS_2, showing two bands in the green at B and F, respectively, and a third in the blue between F. and G. It readily bleached in the light. Millardet believed that the pigment was derived from chlorophyll, but this idea has long since been abandoned.

A. and G. de Negri (1879) regarded the tomato pigment as identical with the rubidin which they isolated from the watermelon. Schimper (1885) observed the red crystals in the ripe tomato fruit, as did also Courchet (1888). Arnaud (1885), however, following his discovery of carotin in the chloroplastids, believed the tomato pigment to be carotin. Passerini (1890) followed Arnaud in this belief and so did Ehring (1896), Tammes (1900) and Kohl (1902). Zopf (1895), however, could not identify it spectroscopically with carotin. Schunck (1903), also, found the red tomato pigment to have a characteristic absorption spectrum. Schunck believed it to be a distinct pigment, different from carotin, and, as previously stated, named it lycopin. The same pigment is found in the leaves of the tomato plant, according to Montanari (1904), but this fact has not been reported by other investigators.

The first hint of the true relation of lycopin to carotin was obtained, however, by Montanari, who submitted the pure crystals to analysis for the first time. He obtained an average composition of C = 88.14 per cent and H = 10.88 per cent, which he regarded as corresponding sufficiently well to the Arnaud formula for carotin, $C_{26}H_{38}$, which was still in vogue at that time. Molecular weight determinations in benzene, using the cryoscopic method, gave values of 635-650, from which fact it was concluded that the pigment was dicarotin, or $C_{52}H_{74}$,

the theoretical molecular weight of which is 698. A melting point of 173.7° C. (corrected) was found for the crystals.

From the work of Willstätter and Escher (1910), however, it is evident that lycopin is identical in general composition and molecular weight with carotin, differing only in solubility in certain solvents and in the position of the absorption bands and in the form of the crystals as well as their color when free and in solution. There is a slight difference in melting point also, lycopin melting between 168° and 169° C., while carotin melts at 167.5° to 168° C. The conclusion that lycopin is a true isomer of carotin seems entirely justified.

The isolation of lycopin was carried out by Willstätter and Escher on their usual generous scale, starting with 74 kg. of tomato conserve, from which 11 grams of pure recrystallized pigment were eventually obtained. Crystals of carotin were also obtained in small amounts as by-product, showing that the factor for yellowing which the red tomato possesses, and which is familiar to the botanist, is due, in part at least, to the usual carotin of the chloroplastids.

The analyses and molecular weight determinations carried out by Willstätter and Escher on the pure lycopin crystals were in excellent agreement with theoretical composition and molecular weight of carotin as found by Willstätter and Mieg (1907), namely, $C_{40}H_{56}$, as shown by the following data.

Calculated for $C_{40}H_{56}$	*Found for lycopin. (Ave. of 4 detns.)*	*Calculated Mol. Wt. for $C_{40}H_{56}$*	*Mol. Wt. found for lycopin. (Ave. of 7 detns.)*
C = 89.48	C = 89.36		
H = 10.52	H = 10.81	536	558

The characteristic properties of lycopin as described by Willstätter and Escher may be summarized briefly as follows. The crystals are dull brownish-red to carmine colored flakes and lack the metallic iridescence of carotin and xanthophyll crystals. The solution in CS_2 retains its bluish-red color on great dilution, and while the ethereal and alcoholic solutions are yellow they have a somewhat browner tone than carotin or xanthophyll solutions. The solubility of the lycopin crystals in the usual carotin solvents, namely, ether, petroleum ether and CS_2, is somewhat less than that of carotin, and it is even more difficultly soluble in hot alcohol than pure carotin. An iodine addition product of constant composition and characteristic form could not be obtained, the product being amorphous and having an iodine content of 34-37 per cent. Lycopin readily oxidizes and bleaches like

the other carotinoids, but the oxidized product has a characteristic, different odor from oxidized carotin, according to Willstätter and Escher. Especially characteristic is the position of the absorption spectral bands of lycopin, particularly in CS_2, three bands being visible, the second of which nearly occupies the space between the first two carotin bands. The measurements as carried out by Willstätter and Escher are as follows, using a 0.05 per cent solution in carbon disulfide. The figures have been confirmed completely by Monteverde and Lubimenko (1913b).

	10 mm. layer.	*20 mm. layer.*	*40 mm. layer.*
Band I	: 554 – –540 μμ	561 – –555 –536 μμ	563 –533 . .525 μμ
Band II	: 514 – –499.4 "	517.5 –498 "	525 –493 . .483 "
Band III	: 479 . .472 "	481.5 – –468 "	483 –462.5 . .427– "

Note: – means very dark; – – means fairly dark; . . means rather weak.

Since Willstätter and Escher's thorough study of the red tomato pigment, Duggar (1913) has observed that green tomato fruits ripened above 30° C. do not form lycopin but only carotin (possibly xanthophylls also), producing a yellow fruit, but that the induced yellow fruits form lycopin if the temperature is reduced to the usual ripening temperatures, namely, 20° to 25° C. These facts are of special interest to the plant physiologist and geneticists.

Of particular interest from the standpoint of those desiring to identify the presence of lycopin in other fruits and plants is van Wisselingh's (1915) study of the microchemical crystallization of lycopin and the effect of various reagents on the crystals thus formed. This investigator finds that lycopin does not readily crystallize in the tomato fruit by the Molisch method at room temperature, but does so more readily at 80° C., and very readily at 140° C., using a 10 per cent solution of KOH in glycerin instead of in alcohol, the high temperature, of course, making the use of alcohol unfeasible. The other carotinoids fail to crystallize at the high temperature. The lycopin crystals which form have a reddish-violet color and show a characteristic color change with bromine from red-violet to blue-violet to blue-green, green, yellow, and finally colorless. Like carotin, the microchemical lycopin crystals are insoluble in phenol-glycerin (3 parts by weight of phenol crystals and one part by weight of glycerin). Van Wisselingh also found what appeared to be carotin crystals in the tomato fruit after carrying out the Molisch procedure at 80° C.

Carotinoids in Seeds and Grains

The wide distribution of carotinoids in flowers and fruits, as revealed in the foregoing paragraphs, naturally justifies the expectation that the same pigments should be found in seeds and especially in the grains of plants of the grass species where fruit and seed are, for practical purposes, one and the same.

Of the true seeds the plant biochemists who have studied pigments naturally have been interested especially in the highly pigmented endocarp or aril which characterizes a number of plants. Several of these have been the object of investigation.

Courchet (1888) recrystallized the ether extractable pigment of the arils of *Euonymous japonicus* (Japanese Spindle-tree), *Momordica Balsamina* (Balsam Apple) and *Passiflora coerulea* (Passion flower plant). The red-orange rhombic shaped tablets obtained from the aril of the Spindle-tree indicate the close relation of the pigment to carotin, while the carmine colored needles which Courchet obtained from the bright red arils of the other two plants were recognized by him as being identical in form and color with those obtainable from tomatoes and from the flesh of watermelons. It would appear that the more orange colored endocarps owe their color to carotin (and probably xanthophylls) while those of a more distinct red color are pigmented by lycopin. This supposition is borne out by the observation of Schrötter-Kristelli (1895a), who found the orange color of the aril of *Afzelia Cuazensis* to be due to carotin, dissolved in a thick orange-yellow oil, from which he recovered and recrystallized the pigment after saponification of the oil. The Toblers (1910a) and Duggar (1913) have confirmed Courchet's observation that the red pigment in the bright red aril of *Momordica Balsamina* is lycopin. Duggar has observed also that the aril pigment of *Momordica charantia* is lycopin, which fact has already been mentioned. Lubimenko (1914a) believes that the aril of *Euonymous Japonicus* owes its color to the same pigment.

There is less certainty regarding the character of the carotinoid in the arils of some of the other plants. Tammes (1900) obtained carotinoid color reactions and a Molisch microchemical crystallization of the pigment in the aril of *Euonymous latifolia* Scop. (Spindle-tree), which was substantiated completely by van Wisselingh (1915). Both Kohl (1902) and van Wisselingh (1915) obtained positive carotinoid reactions for the aril of *Taxus baccata* (Yew tree), but according to

Monteverde and Lubimenko (1913b) this pigment is rhodoxanthin, the red isomer of xanthophyll. True carotinoids are not present in the aril of *Myristica fragrans* Houtt. (Nutmeg), judging from Kohl's (1902) classification of the pigment as a xanthophyll showing no spectroscopic absorption properties. Lubimenko (1914a), however, reports lycopin in the aril of this plant.

Other seeds which are not characterized by highly pigmented endocarpellary tissue have been found to contain carotinoids although nothing is known regarding the distribution of the individual carotinoids among the total pigment. These seeds are characterized by yielding a yellow oil on pressure. Gill (1918) has tested by a carotinoid color test flax seed (*Linium usitatissimum*, the linseed of commerce), mustard seed (*Brassica nigra*) and sesame seed (*Sesamum indicum*), obtaining a positive test; and rape seed (*Brassica campestris*), white sunflower seed (*Helianthus*),[10] and cotton seed (*Gossypium hirsutum*), with negative results. Palmer and Kempster (1919c), however, have found that rape seed increases slightly the color of the egg yolk when fed to laying hens, indicating the presence of some xanthophyll in the seeds. Refined, but unbleached, cottonseed oil is characterized by a rich golden color and Palmer's (1914g) study of the character of the pigments of cottonseed meal has shown that this color is due to a mixture of carotin and xanthophylls. Hemp seed (*Cannabis sativa*) was found by Palmer and Kempster (1919c) to slightly increase the color of egg yolk, and thus appears to contain xanthophyll in small amounts.

The cereal grains also appear to contain carotinoids more or less abundantly. Thudichum (1869) classified the pigment of yellow Indian corn (*Zea mays*) with the luteins. The author's (1914g) study of this pigment, however, shows it to be almost entirely xanthophyll, with a little carotin. Spectroscopically the xanthophyll corresponded with the principal xanthophyll (probably xanthophyll *α*) of the chloroplastids, but its relative solubility and adsorption properties were at variance in that it did not seem to be adsorbed to any extent from petroleum ether or carbon disulfide by $CaCO_3$, and it appeared to be just as readily extracted from 80 per cent alcohol by petroleum ether as from the latter solvent by fresh 80 per cent alcohol. The peculiari-

[10] The particular variety of sunflower seed examined is not clear. The common sunflower (*Helianthus annus*) whose seed is used for commercial oil production, gives a very pale yellow or greenish-yellow oil and it is possible that carotinoids may not be present.

ties of this pigment have not yet been explained and the work should be repeated.

Monnier-Williams (1912) has shown that carotin is one, if not the chief pigment of unbleached wheat flour. The author has confirmed the presence of both carotin and xanthophylls in the wheat grain (*Triticum vulgare*). Carotinoids are also present in small amounts in barley (*Hordeum sativum*) and oat (*Avena sativa*) grains, as shown by Palmer and Kempster (1919c) and even in traces in the grains of polished rice (*Oryza sativa*), as shown by the experiments of the same authors (1919a).

Summary

Carotin, the specific pigment of the carrot root, was first isolated and named by Wachenroder (1826). The hydrocarbon nature of the pigment was discovered by Zeise (1847) and confirmed by Arnaud (1886). The formula $C_{40}H_{56}$ was established for the pigment by Willstätter and Mieg (1907) and confirmed by Euler and Nordenson (1908) and others. Euler and Nordenson showed that xanthophylls are also present in carrots, a fact confirmed by Palmer and Eckles (1914g). Escher (1909) was unable to determine the constitution of carrot carotin although he had at his disposal 150 grams of pure pigment.

Other yellow roots, such as parsnip, sweet potato, yellow turnip, rutabaga, squash, etc., undoubtedly contain carotinoids but the exact nature of the pigments has not been determined.

The existence of yellow pigments in chloroplastids was discovered by Frémy (1860), but the first definite separation from green pigment was made by Stokes (1864), and later by Kraus (1872) and Sorby (1873) and others.

The first crystals of yellow plastid pigment were observed by Frémy (1865) and later by Hartsen (1873a), Bougarel (1877), Borodin (1883) and Guignet (1885). It remained for Arnaud (1885), however, to observe the identity of these crystals with carrot carotin, which was confirmed by chemical analysis through the work of Immendorff (1889) and Willstätter and Mieg (1907).

The plurality of the yellow chloroplastid pigments was first suggested by Stokes (1864a) and definitely demonstrated by Borodin (1883). The correct procedure for the separations of these pigments as well as their present classification as carotinoids was developed by Tswett (1906 to 1911) on the basis of the observations of Kraus

(1872) and Monteverde (1893), as well as on his own physico-chemical studies of the leaf pigments. Tswett's theories regarding the chemical relation between the carotin and xanthophyll groups of carotinoids were substantiated by Willstätter and Mieg (1907), who isolated the first crystalline xanthophyll and established for it the formula $C_{40}H_{56}O_2$. Tswett's adsorption method of analysis of the carotinoids in chloroplastids indicates the existence of at least four yellow xanthophylls accompanying carotin in the leaf. The crystalline xanthophyll isolated by Willstätter and Mieg is probably a mixture of two or more of these xanthophylls. The author proposes a colloidal theory to explain the adsorption method of analysis which reveals the several xanthophyll pigments.

Xanthophylls for the most part are yellow in color, but Monteverde and Lubimenko (1913b) have discovered a red xanthophyll which they call rhodoxanthin.

The types of carotinoids in etiolated plants and their relative proportions have not been studied since the advent of the present carotinoid classification and the development of methods for their separation. A review of the older studies indicates, however, that carotin is concerned in the etiolated color, but the evidence is not clear as to the character and extent of the xanthophyll distribution.

It seems certain that carotinoids are concerned in part in the pigmentation of naturally yellow and yellow spotted leaves. The types of carotinoids and their relative proportions have not been determined by modern methods.

The important questions to be answered regarding the yellow chromolipoids concerned in autumn colorations are: (1) are the yellow autumn pigments merely the carotinoids already present in the chloroplastids, (2) are these augmented or replaced by other yellow pigments closely related to the normal carotinoids but still capable of being differentiated from them, (3) are the yellow autumn pigments entirely new substances? The most recent study of these questions by Tswett (1908c) and Miss Goerrig (1917) shows definitely that the yellow colors are not due to entirely new pigments. It has not been determined with certainty, however, whether or not the chloroplastid carotinoids are slightly modified during the necrobiosis or to what extent new yellow pigments play a part in the autumn colorations. Tswett has concluded that the yellow colors are due entirely to a mixture of slightly modified carotinoids, which he calls autumn xanthophylls, but which the author believes should better have been

named autumn carotins. Green as well as autumn leaves also contain, according to Tswett, colorless water- and alcohol-soluble chromogens which form golden yellow salts with acids and alkalies, particularly the latter, and which readily oxidize to a brown color. These pigments are regarded by Tswett as playing a part at times in the necrobiotic colorations, and the postmortal colors are held to be due entirely to these pigments. Miss Goerrig's conclusions oppose those of Tswett in indicating that the yellow autumn colors are due in part to the normal unchanged carotinoids of the chloroplastids, diminished somewhat in quantity in comparison with the mid-summer green leaves. Miss Goerrig believes, however, that the chief rôle is played by new yellow pigments soluble in water.

Autumn and winter reddening is due at times to red carotinoids. In some cases the red xanthophyll, rhodoxanthin, is involved, e.g., in arbor vitæ. In other cases the red carotin, lycopin, is involved, e.g., certain conifers, under tropical conditions. For the most part, however, red autumn colors are due to anthocyanins.

The vast majority of yellow to orange-red flowers owe their color to chromoplastids containing carotinoids. Very little is known, however, regarding the character and distribution of the individual carotinoids among these flowers. In general, floral colors of a primrose or sulfur-yellow color are produced by water-soluble non-carotinoids which are flavones, anthocyanins or related pigments. The latter are usually present in solution in the cell sap in contrast with carotinoids which are present in plastids. The reader is referred to the tables showing the flowers whose color is due chiefly, if not entirely, to carotinoids.

Carotinoids are undoubtedly the cause of the color of many yellow to orange colored fruits. The reader is referred to the text for the presentation of our present knowledge of this subject. Red tomato fruits are characterized by a red carotinoid called lycopin, which is a chemical isomer of carotin, differing from it only in color and certain physical properties. These relations were recognized first by Millardet (1876) and definitely established by Willstätter and Escher (1910). A. and G. de Negri (1879) first suggested the identity of the watermelon pigment with the red tomato pigment, a supposition finally proved by Monteverde and Lubimenko (1913b). The red pepper pigment is also probably lycopin.

The arils and carpellary tissue of a number of seeds are also characterized by carotinoids, carotin, xanthophylls, lycopin and rhodoxan-

thin having been found in specific cases, which are enumerated in the text. Carotinoids are also found in certain seeds whose carpellary tissue is less highly colored. The cereal grains also contain carotin and xanthophylls. The pigment of yellow maize is characterized by a large proportion of xanthophyll carotinoid.

Chapter III

Carotinoids in the Cryptogams

The non-flowering forms of plant life, the chromolipoids of which are considered in the present chapter, are as abundantly characterized by pigments as the phanerogamous, or flowering forms. Indeed, among the algæ, which will be first considered, the more important classes derive their names, at least their common designations, from their general distinguishing color. The same is true in a few cases for fungi, for example, the rusts.

The information available regarding the character and distribution of carotinoids among the lower forms of plants is, on the whole, more abundant than might be supposed. Speaking first for the algæ, it is surprising to find that our knowledge is practically complete for certain of the classes, particularly the red and brown sea-weeds. On the other hand, fragmentary information only is available for other classes of algæ, so that the subject of the carotinoids among the algæ is by no means as yet a closed book. Some of the algæ seem to owe their characteristic color, at least in part, to carotinoids. This is true of the brown sea-weeds as a class in the living condition. Certain species among other classes apparently owe their color entirely to carotinoid pigments, for example, the so-called blood algae *Haemotococcus pluvialis*, one of the *Chlorophyceae*, but this phenomenon does not seem to be the general rule.

Carotinoid-like colors are more common among the fungi than among the algæ, but the colors in many cases appear to be due to other pigments. In general, it may probably be stated with some degree of assurance that carotinoids are not so common among the fungi as among the algæ. In fact, many fungi appear to be entirely devoid of carotinoid pigment, while practically all classes of algae appear to contain pigments of this type to some extent, or at least to give reactions which may be thus interpreted.

The study of the carotinoids which appear to be regularly produced by bacteria of certain species is practically an unexplored field. Practically nothing is known regarding the character and distribution

of the carotinoids which appear to be produced by certain of these organisms. Splendid opportunities for research exist also in regard to the factors governing the kinds and amount of the pigment produced in each case. Such a study offers some fascinating possibilities in connection with the discovery of the true function of the carotinoids in plants. No matter how acceptable the theories appear to be which are at present in the ascendency regarding this function, it is to be admitted that no theories have been advanced which can claim much experimental basis. It seems logical to assume that much valuable information might be secured if it could be found possible to control the growth and character of carotinoids in simple plants, like the bacteria. If the carotinoids are, after all, merely by-products of plant cell activities we should know this fact. In general, as plant life ascends the scale of complexity carotinoids become an established product of the cell life, and their invariable appearance in the chloroplastids has been interpreted in favor of a functional theory. The fact that the same pigments appear at times in other organs of the chlorophyllous plants and also in plants which lack chlorophyll entirely may, however, be significant. At any rate the possibility is not to be overlooked of throwing some light on this question through a study of the carotinoids in bacteria.

Carotinoids in the Algae

The plan which will be followed will be to present the available knowledge regarding each class of algae separately. The species which have been examined will be tabulated, together with the names of the investigators and the dates their work was published. The author has found such an arrangement helpful in the study of the subject and believes it will furnish a convenient mode of reference for future workers in this field. The order of presentation of the various classes follows in general a descending scale with regard to complexity of the plant forms.

The Phæophyceae. These plants, commonly known as the brown or olive-brown sea-weeds, comprise a large group, which are mostly marine plants. They are found everywhere in the seas, especially in the colder waters. A few of the species are of economic importance. *Laminaria saccharina,* which contains the carbohydrate mannite, is used in the Orient for food. The carotinoids of this class of algae are better known both qualitatively and quantitatively than the chromo-

lipoids of any other class of algae. The fresh plants owe their olive-brown tint to the high concentration of the special algæ carotinoid, fucoxanthin, the most recent member of the chromolipoid pigments to be brought to a definite chemical conception. The dried plants owe their brown color to the pigment phycophäin, which forms in the plants after death, as the result of oxidation of colorless chromogens in the cells. The belief that phycophäin is the characteristic coloring matter of the brown algae still finds expression in the text books, but this is only true of the dried plants.

The brown sea-weeds which have been examined for carotinoids are given in Table 8. The table shows that the pigments of these plants attracted the attention of the early workers. While the scope of these first studies was naturally limited by the prevailing knowledge of the plant chromolipoids, they unquestionably paved the way for the discovery of the fucoxanthin which characterizes the brown algae. The fact that certain species, like the *Fucoidiae* and *Laminaria,* are abundant and easily obtained no doubt accounts for their popularity as sources of material for investigation. Of the various investigations mentioned in the table those of Tswett (1906), Czapek (1911), Kylin (1912) and Willstätter and Page (1914) are the most important. The chief contributions of these, as well as the earlier investigators, to the subject may be summarized as follows:

Rosanoff (1867) appears to have first expressed the belief that the *Fucoidiæ* contain a special pigment besides chlorophyll.

Millardet (1869), working in Kraus' laboratory, submitted the absolute alcohol extracts from several species of both the dried and fresh plants to the benzene separation method which had just been worked out by Kraus. The yellow pigment remaining in the alcohol layer was regarded as differing from the xanthophyll of higher plants and was called phycoxanthin, the name which Kraus and Millardet (1868) had already given to the pigment prepared in the same manner from green algae and diatoms. Millardet was also the discoverer of the brown water-soluble pigment of the *Phæophyceæ,* to which the name phycophäin was given. Reinke (1886) first expressed the belief that phycophäin is a post-mortem product. Molisch (1905) first offered experimental proof of this fact which was later definitely proved by Tswett (1906) and confirmed by Kylin (1912).

Askenasy (1869) noticed that the yellow pigment discovered by Millardet in the brown algæ turns blue when its alcoholic solution is treated with HCl, a reaction which appears to be specific for fuco-

xanthin, and is of considerable importance for the qualitative detection of this pigment.

Sorby (1873) proposed the name fucoxanthin for the chief coloring matter of the brown algae. He differentiated the pigment from the xanthophyll of the higher plants by reason of the position of its spectroscopic absorption bands, by its greater resistance to the bleaching action of light, and by the blue color obtained when HCl is added to the alcoholic solution of the pigment, all of which characteristic properties have since been confirmed for the pure pigment. Sorby also isolated "orange xanthophyll" (carotin) from brown algae, and was thus the first to show the presence of the better known carotinoids in these plants.

Reinke (1876) proposed the name phäophyll for the special yellow pigment of *Phæophyceæ.*

Hansen (1884d) regarded the yellow pigment of brown algae as identical with the "yellow chlorophyll" of higher plants.

Tammes (1900) demonstrated the presence of carotinoids in a number of species of *Phæophyceæ,* using the Molisch alkali microchemical crystallization method. It is now known that he was mistaken in attributing the result to carotin only.

Gaidukov (1903), however, denied that either carotin or a special pigment, phycoxanthin (using Millardet's terminology), characterizes the brown sea-weeds, claiming to have found in addition to chlorophyll only the xanthophylls which characterize the higher plants.

TABLE 8. PHAEOPHYCEAE FOUND TO CONTAIN CAROTINOIDS

Order *Cyclc*[illegible]rms).
- *F*[illegible] 1867; Czapek, 1911.
- *Fucus serratus*—Millardet, 1869; Sorby, 1873; Reinke, 1876; Tammes, 1900; Gaidukov, 1903; Molisch, 1905; Tswett, 1905, 1906; Kylin, 1912; Willstätter and Page, 1914; van Wisselingh, 1915.
- *Fucus vesculosus*—Millardet, 1869; Hansen, 1884; Tammes, 1900; Tswett, 1906; Kylin, 1912; van Wisselingh, 1915.
- *Fucus nodosus*—Millardet, 1869.
- *Fucus versoides*—Molisch, 1905.
- *Ascophyllum nodosum*—Tammes, 1900; Kylin, 1912; van Wisselingh, 1915.
- *Phyllospora Brodiaei, P. membranifolia*—Kylin, 1912.
- *Cystoseira abrotanifolia*—Molisch, 1905.
- *Halidrys siloquosa*—Millardet, 1869; Reinke, 1876; Molisch, 1905; Kylin, 1912.

Order *Phæosporales.*
- *Ectocar*[illegible] 1869.
- *Pylaiell*[illegible], 1912.
- *Desmarestia aculeata*—Reinke, 1876.
- *Elachista* species—Millardet, 1869; Molisch, 1905.

Leathesia marina—Millardet, 1869; Molisch, 1905.
Chorda filum—Tammes, 1900.
Laminaria saccharina—Millardet, 1869; Reinke, 1876; Tammes, 1900; Molisch, 1905; Tswett, 1905, 1906; van Wisselingh, 1915.
Laminaria digitalis—Tammes, 1900; Molisch, 1905; Kylin, 1912; Willstätter and Page, 1914; van Wisselingh, 1915.
Cutleria multifida—Millardet, 1869; Molisch, 1905.

Order *Dictyotales*.
Dictyota dichotoma—Millardet, 1869; Molisch, 1905.
Dictyopteris polypodioides—Tammes, 1900; Molisch, 1905; Kylin, 1912.
Halyseris polypodioides—Millardet, 1869; Molisch, 1905.
Padina Pavonia—Molisch, 1905.

Molisch's (1905) contributions to the carotinoids of brown algae were, (1) in showing that the water-soluble phycophäin exists only in the dried plants or those which have been placed in hot water for a few minutes, (2) in rediscovering the blue color reaction with HCl. Molisch obtained the latter reaction either by extracting the fresh plants with alcoholic-HCl (98 volumes of alcohol and 2 volumes of con. HCl) or by adding HCl to the alcoholic layer after the Kraus separation, using petroleum ether for extracting the carotin and chlorophyll. Molisch, however, did not attribute this reaction to a carotinoid, but to a colorless "leucocyan" in the plant, which gave rise to a blue "phaeocyan" with HCl.

Tswett (1905) was quick to point out Molisch's error with respect to the so-called leucocyan reaction, showing that this was due to the special carotinoid, fucoxanthin, in the plants, as Sorby (1873) had pointed out many years earlier. Tswett (1906) was thus led to make a closer study of the *Phaeophyceae* pigments, using *Fucus* and *Laminaria* for his material. He showed first that phycophäin is a post-mortem *oxidation* product and does not exist in the living plants. He next made a careful examination of the chromatophor pigments of the living plants making use of the relative solubility and chromatographic adsorption properties of the carotinoids. By this means he showed definitely that at least three carotinoids are present, namely, fucoxanthin, carotin, and xanthophyll. The former is the principal pigment. It corresponds to the xanthophylls of the higher plants in being adsorbed from pure petroleum ether and carbon disulfide by calcium carbonate and other finely divided materials, and by remaining hypophasic in the Kraus separation between petroleum ether and 80 per cent alcohol. It differs from the xanthophylls of the higher plants in the position of its absorption bands in the spectrum, by the reddish-brown color of its concentrated solutions, by the fact that it is attacked

by alkalies, and by its color reaction with HCl. Tswett noticed, like Sorby, that alkali would restore the yellow color of alcoholic solutions turned blue with acid, but mentioned that the shade of yellow was not quite the same as the original. Tswett regarded the carotin present as identical with the carotin of higher plants. The xanthophyll, however, was probably erroneously regarded as a special xanthophyll, to which Tswett gave the name fucoxanthophyll. Tswett also studied the chlorophyllins of the brown algae, finding chlorophyllin α (chlorophyll a of Willstätter) and chlorophyllin γ, a special pigment, which he regarded as characteristic of the *Phæophyceæ*. This has not been confirmed by Willstätter and Page (1914) who found only chlorophyll a in the brown algae.

Czapek (1911) submitted petroleum ether extracts of carefully dried *Fucoideæ* to a Tswett chromatographic analysis and found chlorophyll a, fucoxanthin and xanthophyll, but no carotin. The failure to find carotin was probably due to the fact that *Laminaria*, according to Willstätter and Page, contain very small quantities of carotin.

Kylin (1912) has given us one of the best systematic examinations of the carotinoids of the *Phaeophyceae*. Although Kylin falls into the error of regarding the Molisch microchemical crystallization test, which he performed on a large number of species, as specific for carotin, he nevertheless succeeded in isolating the first crystals of this pigment from brown algae. Impure crystals of xanthophyll were also secured. An unsuccessful attempt was also made to secure crystals of fucoxanthin, for which pigment Kylin prefers the name phycoxanthin. Kylin pointed out the probable close chemical relation of fucoxanthin to xanthophylls. He found that a greater solubility in petroleum ether is one of the distinguishing differences, a result which Willstätter and Page (1914) find is characteristic of the impure pigment, but not of the pure crystals. The latter are insoluble in petroleum ether. Kylin made the interesting discovery that the blue color reaction is given not only by the mineral acids, but by acetic and oxalic acids as well, and that dilute alkali changes the pigment so that the tendency to give this reaction is greatly accelerated.

Fucoxanthin. The chemical relation of fucoxanthin to the other carotinoids is now known through the work of Willstätter and Page (1914), who also determined the quantitative distribution of the different carotinoids in the olive-brown sea-weeds. Ultimate analyses

carried out on five different preparations of fucoxanthin gave the following results in comparison with several theoretical values.

Found	Calculated for		
	$C_{40}H_{54}O_6$	$C_{40}H_{56}O_6$	$C_{41}H_{56}O_6$
C = 76.39 H = 8.77	76.14 8.63	75.90 8.93	76.35 8.76

The investigators prefer the formula $C_{40}H_{54}O_6$ over $C_{40}H_{56}O_6$, although the latter expresses the closer empirical relation to carotin, but admit, at the same time, that their data correspond most closely to the formula containing one additional carbon atom. In any case the close chemical relation of fucoxanthin to the other carotinoids is clearly established.

The observations of Tswett and of Willstätter and Page show that great care must be taken in the isolation of fucoxanthin to prevent the formation of the post-mortal phycophäin. This was prevented by Willstätter by dehydrating the fresh plants with 30 per cent acetone, after which the material was macerated and extracted at once with pure acetone. The extract containing the combined chlorophyll and carotinoids was then diluted with ether which was washed free from acetone with water. The ether extract was now diluted with an equal volume of low boiling petroleum and the mixture submitted to a modified Kraus separation using 70 per cent methyl alcohol. In this connection Willstätter and Page made the valuable observation that fucoxanthin is quantitatively removed by 70 per cent alcohol in the Kraus separation, leaving the other carotinoids practically quantitatively in the petroleum ether, especially if ether is also present. It is clear that this discovery not only permits the separation of fucoxanthin but does not interfere with the subsequent separation of xanthophyll and carotin by the usual procedure using petroleum ether and 80 per cent methyl alcohol.

Fucoxanthin isolated on the above principle was found to crystallize readily from methyl alcohol or acetone in dark red regular hexagons, containing water or alcohol of crystallization, the latter being lost only in high vacuum at 105° C. By precipitating the pigment from ether with low boiling petroleum, in which it is insoluble, compact needles without any solvent of crystallization were obtained.

A study of the characteristic color reaction with HCl showed it to be

due to the formation of a hydrochloride of the composition $C_{40}H_{54}O_6$, 4HCl, the yellow pigment restored by alkali still containing one molecule of HCl. The instability of the pigment in the presence of strong alkali, which was observed by Tswett, was confirmed on the pure substance, it being shown that a new substance was formed which, when set free from the alkali by water, showed characteristic spectroscopic and solubility properties. It was found that the effect of alkali in increasing the sensitiveness of fucoxanthin solutions towards the blue color reaction with acids, which Kylin noted, was due to this modified pigment. Willstätter and Page observed that as little as 0.001 per cent HCl would give the blue color with a concentrated ether solution of the pigment, after its modification with alkali.

Fucoxanthin appears to be an even more intense pigment than carotin or xanthophyll from the observations of Willstätter and Page, who found that 85 mm. of 0.2 per cent $K_2Cr_2O_7$ has the color equivalent of 108 mm. of xanthophyll, or 80 mm. of carotin, but only 50 mm. of fucoxanthin, using in each case a 5×10^{-5} molar concentration of pigment.

Other interesting properties of fucoxanthin observed by these investigators were the formation of oxonium salts, a crystalline iodide, $C_{40}H_{54}O_6I_4$, and a bleached oxidation product $C_{40}H_{54}O_{16}$. In connection with the last named product it was found that the crystals of pigment are much more stable than carotin or xanthophyll, but that the solutions, especially benzene solutions, bleach readily.

Regarding the quantitative distribution of the carotinoids in *Phaeophyceae,* Willstätter and Page give the following figures on both the fresh and dry basis for species representing the three principal orders of these plants.

	Fresh Algæ			Dry Algæ		
	Fucoxanthin per cent	*Carotin* per cent	*Xanthophyll* per cent	*Fucoxanthin* per cent	*Carotin* per cent	*Xanthophyll* per cent
Fucus	0.0169	0.0089	0.0087	0.0593	0.0312	0.0305
Dictyota	.0250	.0057	.0063	.....	.....	.....
Laminaria	.0081	.0006	.0038	.0528	.0038	.0243

Willstätter and Page gave some attention to the character of the xanthophyll present. They were unable to observe any properties which would serve to distinguish the xanthophyll isolated by them from the crystalline xanthophyll of higher green plants. This result throws doubt on the existence of a special fucoxanthophyll in the

brown algæ, as Tswett concluded. The question still remains open, however, as to whether more than one xanthophyll is present.

The Rhodophyceae. These plants, commonly known as the red seaweeds, are, like the brown algae, found mostly in salt waters, only a few inhabiting fresh water. Both forms, fortunately, have been examined for carotinoids. The plants are especially abundant in the tropic oceans and in the temperate regions at lower depths. Several hundred species have been described. The dried thallus of *Chondrus crispus* forms the carragheen, or dried moss which is used for its gelation properties. Various species of these plants are the source of agar-agar. The thallus of the *Rhodophyceae* is abundant in pigment and may be red, violet or purple, but rarely green. The characteristic pigment, however, never appears to be carotinoid. Some chlorophyll appears to be present, but the chief pigment is a protein-like material or is combined with such (Kylin, 1911), and is known as phycoerythrin.

The red algae, however, do not lack carotinoids. Those in which the chromolipoids have been demonstrated are given in Table 9. The investigations upon which our knowledge of the carotinoids in the red algæ depends may be summarized as follows.

Sorby (1873) appears to have first called attention to the presence of yellow pigments in this class of plants, when he was able to demonstrate the presence of xanthophyll in *Porphyra vulgaris*. It will be recalled from the summary of Sorby's work given in Chapter II that his "xanthophyll" corresponds in properties with the xanthophyll *a* of the higher plants as revealed by Tswett's chromatographic analysis, and also to the chief properties of the crystalline xanthophyll isolated from green plants in Willstätter's laboratory.

Reinke (1876) extracted *Batrachospermum moniliforme* with hot alcohol, and obtained a yellow extract which gave up its pigment to benzene. This result indicates carotin in the light of our present knowledge of the relative solubility properties of the carotinoids.

Nebelung (1878) examined the effect of alcohol and petroleum ether as solvents for the pigments of several fresh water *Rhodophyceae,* and found that a yellow pigment (or pigments) could be extracted.

Hansen (1893) applied his method of separating green and yellow pigments to the alcoholic extracts of a number of red algae. He found that green and yellow fractions could be obtained by treating the extracts with alkali and shaking with ether. He regarded the

result as indicative of the same types of pigments as are present in higher plants which he had studied with like results.

Tammes (1900) demonstrated carotinoids in three species of red algae, which are mentioned in Table 9, using the Molisch micro-crystallization method. Kohl (1902), using the same method, confirmed this work as well as obtaining positive results on other species.

TABLE 9. RHODOPHYCEAE FOUND TO CONTAIN CAROTINOIDS

Order *Bangiales*
- *Bangia* species (fresh water)—Nebelung, 1878; Kohl, 1902.
- *Porphyra laciniata*—Tammes, 1900.
- *Porphyra hiemalis*—Kylin, 1911.
- *Porphyra vulgaris*—Sorby, 1873.

Order *Nemalionales*
- *Lemania fluviatilus* (fresh water)—Nebelung, 1878; Kohl, 1902.
- *Batrachospermum moniliforme* (fresh water)—Reinke, 1876; Nebelung, 1878; Kohl, 1902.
- *Chantransia* species (fresh water)—Nebelung, 1878; Kohl, 1902.

Order *Gigartinales*
- *Chondrus crispus*—Kylin, 1911; van Wisselingh, 1915.
- *Cystoclonium purpurascens*—Kylin, 1911.

Order *Rhodymeniales*
- *Dellesseria* [illegible] 1911.
- *Laurencia* [illegible] 1911.
- *Polysiphon*[illegible], 1900.
- *Polysiphonia nigrescens*—Kylin, 1911.
- *Rhodomela subfusca, R. virgata*—Kylin, 1911.
- *Ceramium rubrum*—Tammes, 1900; Kylin, 1911; van Wisselingh, 1915.
- *Ceramium* [illegible] 1911.
- *Callithamn*[illegible] 1911.
- *Spermothar*[illegible] 1911.

Order *Cryptonemiales*
- *Dumontia filiformis*—Kylin, 1911.
- *Furcellar*[illegible] 1911.
- *Polyides* [illegible] 1911.
- *Corallina* [illegible] 1911.

Kylin (1911) has given us the most complete study of the carotinoids of the *Rhodophyceae*. He first successfully applied the Molisch carotinoid test to some 18 different species of red algæ, as noted in Table 9. Unfortunately this test is not specific for carotin as Kylin believed. *Ceramium rubrum* was employed for a special study of the carotinoids. Both carotin and xanthophylls were demonstrated by applying the Kraus procedure to extracts of the plants. There seems to be no question regarding the presence of carotin in the red algæ. The study of the xanthophylls led to less satisfactory results. By evaporating the xanthophyll-containing alcohol fraction to dryness and heating the residue with petroleum ether it was found possible to separate the pigment into two fractions. The fraction which dis-

solved was readily re-extracted from the solution by 80 per cent alcohol, and its solubility in petroleum ether was the only point of difference observed between the two xanthophylls. Spectroscopic and adsorption properties were not examined. Both xanthophylls turned green and then blue on addition of acids to their alcoholic solutions and alkali restored the yellow color. This property is characteristic of Tswett's xanthophyll β of higher plants, as pointed out in the preceding chapter. The reaction also resembles the color reaction of fucoxanthin with acids. Kylin, himself (1912), suggests this in a footnote of the report of his study of the pigments of the brown algae, but was unable to decide whether the color reaction was due to traces of brown algae and diatoms present with his material, or to the actual presence of fucoxanthin in the red algae. It is doubtful whether Kylin really effected a separation of distinct pigments in his xanthophyll fractions. It is not at all unlikely that the *Rhodophyceæ* contain some fucoxanthin. A spectroscopic and chromatographic analysis of the xanthophylls of the red algae as well as an application of the modified Kraus procedure for separating fucoxanthin from the other carotinoids would be helpful in deciding whether the color reaction observed by Kylin was due to fucoxanthin or to a xanthophyll of the β type.

Van Wisselingh (1915) has recently demonstrated carotinoids in two species of red algae using both the Molisch and the acid microcrystallization method.

The Charales. This class of algae, commonly known as the stoneworts, have the interesting property of depositing calcium from the waters in which they thrive, from which they derive their popular name. Only two genera are known, namely, *Chara* and *Nitella*. Both Tammes (1900) and Kohl (1902) were successful in showing carotinoids to be present in *Chara fragilis,* using the Molisch method. The same result was obtained by van Wisselingh (1915) on *Nitella* spores. The evidence points to the presence of carotinoids in the stoneworts, but nothing further is known regarding their character.

The Chlorophyceae. The so-called green algae constitute one of the largest and most important classes of lower plants. They are found in both fresh and salt water, but the former predominate. The cells as a rule contain chloroplastids which makes the question of the types of plastid pigments present an important one. The feature which has especially attracted attention, however, is the fact that the spores

of certain species, e.g., *Haematococcus*, in the resting stage are characterized by a deep red or violet pigmentation. The species which have been examined for carotinoids represent all the important orders. These are given in Table 10.

The development of the question of the character of the pigments other than chlorophyll which characterize the green algæ has followed closely the development of the ideas regarding the carotinoids in the higher plants, as given in Chapter II. For example, the earliest workers, i.e., Cohn (1850), DeBary (1856), Caspary (1858), Hildebrand (1861) and Frank (1877) observed merely that the yellow or red pigments present in the algae which they examined responded to extraction by the fat solvents and gave either the blue color reaction with concentrated H_2SO_4, characteristic of the pigments later known as lipochromes, or the blue color with iodine which also characterizes these pigments. The spectroscopic studies of Nebelung (1878) unfortunately contributed very little to the elucidation of the character of the pigments present. Klebs (1881) made a more thorough study of the properties of the "yellow oil" in certain species, and mentions properties now well recognized as class characteristics of the carotinoids.

Borodin (1883) appears to have first definitely recognized the relation of the yellow pigments of the *Chlorophyceae* to those of higher plants and isolated red rhombic carotin crystals from *Spirogyra*. Molisch (1896), Tammes (1900) and Kohl (1902) as well as van Wisselingh (1915) have also demonstrated the presence of carotinoids in a number of species using the Molisch micro-crystallization test.

Rostafinski (1881) first expressed the possibility of xanthin (older terminology for carotin) being present in the species of green algae which he examined. Willstätter and Page have not only shown that this is the case for *Ulva lactuca* but have determined both carotin and xanthophyll quantitatively in this species. The amounts found were 0.0243 grams of carotin and 0.0643 grams of xanthophyll per kilo of fresh material. The interesting point here is that practically the same proportion between carotin and xanthophyll is found to exist in this species of algae as in the leaves of the flowering plants.

Attention has already been called to the interesting phenomenon of the red color of certain species of *Chlorophyceae* in the resting stage. Special study of the character of the pigments present in *Sphaerella* (*Haematococcus* = *Chlamydococcus*) *pluvialis*, the so-

called blood algæ, and *Trentepohlia Jolithus*, the so-called violet algæ, has been made by Rostafinski (1881), Karsten (1891) and Zopf (1892a, 1895). Cohn (1850) first called the pigment haematochrome and this name was adopted by a number of subsequent investigators for the red pigment of many species of green algae. Rostafinski attempted to associate the haematochrome with chrysoquinone, because the latter, like the red algae pigment, gave the blue color reaction with concentrated H_2SO_4. A spectroscopic comparison of the two pigments failing to substantiate such an identity the name chlororufin was proposed for the algae pigment, and the possibility expressed of an identity with the solanorubin (lycopin) which had been described a few years previously by Millardet (1876). Karsten observed that the haematochrome extracted from *Trentepohlia* by absolute alcohol stained brown with osmic acid, a reaction, however, which may have been due to traces of fat present in the extracts.

TABLE 10. CHLOROPHYCEÆ FOUND TO CONTAIN CAROTINOIDS

Order *Oedogoniales* (highest forms).
Oedogonium species—Tammes, 1900; Kohl, 1902; van Wisselingh, 1915.
Bulbochaete—DeBary, 1856; Cohn, 1867.
Bulbochaete setigera—Kohl, 1902.

Order [illegible]
[illegible], 1881.

Order *Conjugatæ*
Spirogyra crassa—Borodin, 1883; Molisch, 1896; Tammes, 1900; Kohl, 1902.
Spirogyra maxima—Borodin, 1883; van Wisselingh, 1915.
Zygnema cruciatum—van Wisselingh, 1915.

Order *Ulotrichales*
Phycopeltis epiphyton. P. Teubii, P. maritima, P. aurea, P. amboinemis—Karsten, 1891; Zopf, 1892a.
Ceph[illegible] *laevis, C. solutus, C. albidus, C. parasiticus, P.* [illegible] Karsten, 1891; Zopf, 1892a.
[illegible] *maxima, T. moniliformis, T. crassiaepta, T.* [illegible] 1891; Zopf, 1892a.
[illegible]; Zopf, 1892a; Kohl, 1902.
Trentepohlia aurea—Rostafinski, 1881; Zopf, 1892a.
Trentepohlia umbrina—Caspary, 1858; Frank, 1877; Zopf, 1892a.
[illegible] *ureum-tomentosum*—Caspary, 1858; Hildebrand, 1861. [illegible] Cohn, 1867.
Stichococcus majus—van Wisselingh, 1915.

Order *Ulvales*
Ulva lactuca (Sea lettuce)—Willstätter and Page, 1914.
Enteromorpha intestinalis—Tammes, 1900.

Order *Siphonocladiales*
[illegible] *glomerata*—Nebelung, 1878; Tammes, 1900; Kohl, 1902; van Wisselingh, 1915.
Sphaeropleacea—Cohn, 1867.

Order *Siphonales*
Vaucheria species—Nebelung, 1878.
Chlorella protothecoides, C. variegata—van Wisselingh, 1915.

Order *Protococcales* (lowest forms, unicellular)
Sphaerella (*Haematococcus* or *Chlamydococcus*) pluvialis—Cohn, 1850, Rostafinski, 1881; Klebs, 1883; Zopf, 1895; Kohl, 1902; Jacobsen, 1913; van Wisselingh, 1915.
Volvox—Cohn, 1867.
Protococcus (*Pleurococcus*) *pluvialis*—Cohn, 1850, 1867.
Protococcus vulgaris—van Wisselingh, 1915.
Scotinosphaera paradoxa—Klebs, 1881.
Phyllobium dimorphum, P. incertums—Klebs, 1881.
Hydrodictyon utriculatum (water-net)—Tammes, 1900.

In Zopf's (1892a) first study of haematochrome it was pointed out that the previous investigations of the pigment were made on impure mixtures. Zopf not only succeeded in isolating practically pure crystals of the pigment from *Trentepohlia Jolithus,* but also established their identity in form and properties with the carotin from carrots. Zopf later (1895) was led to compare the blood and violet algae from a pigment standpoint. It was found that the fresh vegetation of the latter presents a brighter red appearance, the cells under the microscope appearing yellow to orange; *Sphaerella pluvialis,* on the other hand, appears dark red-brown when fresh, even thin layers in water having a blood red color, the same coloration appearing under the microscope. These and other differences led to a special examination of what appeared to be a special pigment in *Sphaerella* (*Haematococcus*) *pluvialis* different from the carotin in the *Trentepohlia.* Two carotinoids were found, namely, carotin proper, which Zopf called "eucarotin," and a red carotinoid-like pigment which is called "carotinin," the latter being the predominating pigment.

Special interest attaches to this red "carotinin." Its properties, as described by Zopf, are characterized by combining readily with alkalies and by showing only one wide spectroscopic absorption band at the F line. In other respects it has the class characteristics of a carotinoid. Some doubt, however, is thrown upon the alleged alkali combinations of the red "carotinin" described by Zopf by the observations of van Wisselingh (1915), who made a special study of the response of the blood algae to microchemical crystallization tests, as well as the effect of various reagents on the pigment crystals. Three carotinoids were found. The Molisch alkali method applied to the green spores of the algae gave red platelets insoluble in phenol-glycerin (a xanthophyll solvent) and orange needles soluble in phenol-glycerin, indicating the presence of carotin and xanthophyll. The red spores when treated with the Molisch reagent gave red-violet crystal aggregates which contained two pigments, one an orange

yellow and the other a violet colored substance. The latter was regarded as Zopf's red carotinin. Its xanthophyll nature was shown by its ready solubility in the phenol-glycerin reagent. The possibility of this pigment being a potassium compound as it should be from Zopf's description, inasmuch as it was produced in a strongly alkaline medium (Molisch's reagent), was tested by treating the crystals with dilute acid for 24 hours. No change was produced in their properties.

The red pigment of the blood algae deserves further study in the light of the apparently conflicting observations of Zopf and van Wisselingh. Its red color and xanthophyll-like properties as described by the latter investigator suggests the red rhodoxanthin described in Chapter II. No other carotinoids have yet been described, however, which show acid properties and combine with alkalies as the red algae pigment is stated to do.

An interesting observation made by van Wisselingh in connection with his study of the blood algae was that the plants had mostly green aplanospores when cultivated in media containing 0.01 per cent each of KNO_3, $(NH_4)_2HPO_4$, $MgCl_2$, Na_2SO_4, but that the aplanospores were mostly red when allowed to develop in media containing 0.02 per cent NH_4NO_3, K_2HPO_4 and $MgSO_4$. Jacobsen (1913) has also studied the conditions governing the formation of pigment in *Haematococcus pluvialis* and found that temperature as well as food conditions influence it. He was unable to extract the pigment from the plant with fatty oils, and it did not respond to Tswett's resorcin method for the microcrystallization of carotinoid.

The Bacillariea (*Diatomaceae*). The diatoms are unicellular algae of very peculiar structure and interesting habits. The single cells are composed of two symmetrical valves which are held together by a membranous sac of slightly colored protoplasm. The single cells are 10μ or less in diameter. The valves of which they are constructed are frequently beautifully sculptured, and when many of the cells unite, as is sometimes the case, very peculiar shaped structures often result. The epidermis of the diatoms is composed of silica which these organisms have the power to extract from the water in which they develop. Deposits of silica from great growths of these plants have considerable commercial value as diatomaceous earth. The algæ inhabit stagnant water, wet rocks and the sea.

The diatoms comprise a considerable portion of the plankton of the sea. It is this fact, together with the part which the plankton of the

sea plays in the food of marine animals which makes the pigments of the diatoms of interest. Most species of diatoms have a brownish color. A few are green and probably contain chlorophyll, or at least one of the chlorophyll pigments. Of the many thousands of species which are known, unfortunately only a few have been examined for carotinoids. However, these are probably to be considered as typical of the remainder.

The earliest workers regarded the color of the diatoms as due to a single pigment to which Nägeli (1849) gave the name diatomin. This name was adopted by Askenasy (1867) for the brownish yellow pigment which could be extracted with alcohol, and which he described as showing a strong absorption of the blue half of the spectrum and a characteristic intense blue-green color on addition of H_2SO_4 or HCl to the alcoholic solution. Nebelung (1878) extracted a yellow pigment from *Melosira* species with petroleum ether but called it phycoxanthin. The principal pigment extracted in this case may have been carotin.

Further proof of the carotinoid nature of the *Bacillarica* pigments was furnished by Tammes (1900), who obtained the Molisch test on *Fragilaria* species and by Kohl (1902) who obtained the same test on *Gomphonema* and *Navicula* species. Molisch (1905), himself applying the test to *Nitzschia Palea, Nitzschia sigmoidea, Cymatopleura solea* and *Pinnularia viridis* (*Navicula viridis*), obtained only yellow drops, but these gave the chromolipoid color reactions with H_2SO_4 and iodine.

The conclusions of the various investigators are somewhat conflicting regarding the exact nature of the carotinoids present in the siliceous algæ. Zopf (1900) concluded that "eucarotin" (true carotin) is the chief pigment present in *Gomphonema,* but that the pigment differs somewhat from the carotin of other plants. Kohl (1906a) concluded that the liver-colored diatoms, *Achnanthidium lanceolatum* and *Eunotia* (*Himanthidium*) *pectinalis,* owe their color chiefly to carotin with a little of his so-called β-xanthophyll (which is not carotinoid in the true sense) present also, as well as traces of chlorophyll. Kohl had previously (1902) concluded that the pigment known as diatomin is carotin. Especially interesting is the observation of Molisch that the species which he examined (1905) gave the so-called leucocyan reaction which is apparently specific for fucoxanthin. Askenasy (1867) had observed the same reaction for alcoholic extracts of diatoms, so that there are at least strong indications that

fucoxanthin is present in these algae. The view of Kohl (1906) that the leucocyan reaction is specific for carotin is hardly to be regarded as tenable. The imperfect studies which have been made do not indicate whether true xanthophylls are also included among the carotinoids of the *Bacillariea,* but it is not unlikely that this will be found to be the case when the matter comes to be examined in detail.

The Peridinieae. The *Peridiniales,* also called the *Dinoflagellata* comprise a relatively small class of unicellular algae, which are found mostly in salt (sea) water. They sometimes form an important part of the plankton of the sea, so that their pigments are of interest, as in the case of the diatoms, on account of the part which the plankton of the sea plays in the food of fishes and other marine animals.

Schütt (1890) appears to have made the only specific examination of the pigments of the *Peridinieae,* but since his work was performed before the most important developments took place in the field of carotinoids it is necessary to interpret his observations in the light of present-day knowledge of the subject. Up to his time the color of the Dinoflagellates was regarded as due to the same pigment which was believed to color the diatoms, namely, diatomin. As is now known, diatomin is not a specific pigment. Although Schütt did not recognized this fact he did point out that the color of the *Peridinieae* is more reddish-brown and easily distinguished from the yellowish-brown color of the diatoms. This difference in tint was found to be due to the presence of carmine colored drops or globules in many of the *Peridinieæ* examined, in addition to the yellowish-brown pigment in the chromatophors of the algae.

The *Peridinieæ* examined by Schütt were *Gymnodinium Helix, Dinophysis acuta, D. laevis, Certium tripos, C. fusus, C. furco, Peridinium divergens, Prorocentum micans,* and *Glenodinium* species. In addition to brownish-red and brownish-yellow water extracts, the pigments of which were regarded as analogous to the phycoerythrin of the *Rhodophyceae* and the phycophäin of the *Phaeophyceae,* respectively, a wine-red alcohol extract was obtained. The pigment thus extracted, which could not have been pure, was soluble in benzene, ether, chloroform, carbon disulfide, and glacial acetic acid, but very little soluble in petroleum ether. Schütt regarded the pigment as analogous to diatomin, and called it peridinin. The slight solubility of the pigment in petroleum ether and ready solubility in alcohol suggests that a xanthophyll-like pigment predominates in the *Peridinieae.* It is not possible to draw more specific conclusions than

this on such meager data. The writer is of the opinion that examination will disclose the fact that fucoxanthin or a similar pigment is the predominating carotinoid in the *Peridinieae.*

The Flagellata. The flagellates are simple unicellular, aquatic organisms intermediate between the algæ and protozoa. They inhabit ponds and streams. Only a few species have been examined for pigments. A survey of the somewhat scanty evidence does, however, point with certainty to the presence of carotinoids in those species which have been examined. The exact character of the carotinoids remains to be determined.

Wille (1887) regarded the pigment in the brown palmella-like cells of *Chromulina* (*Chromophyton*) *Rosanoffii* as diatomin because the cells turned green when treated with HCl. Klebs (1893) expressed the same idea for *Chrysomonidina* Stein, but called the pigment chrysochrome. Gaidukov (1900), however, emphatically denied the existence of either carotin of fucoxanthin in *Chromulina,* claiming to have found only two pigments present, a chlorophyll-like pigment (chrysochlorophyll) and a xanthophyll-like pigment (chrysoxanthophyll). The latter pigment as described by Gaidukov shows true xanthophyll properties, except that only one spectroscopic absorption band was observed, namely, at 495-485$\mu\mu$, which corresponds fairly well with the first xanthophyll band. Spectroscopic studies of alcoholic and petroleum ether extracts of *Hydrurus penicillatus* were made by Nebelung (1878), but his results give very little hint as to the true character of the carotinoids present.

Two species of *Flagellata* whose pigment has long been of interest are *Euglena sanguinea* and *Euglena viridis.* In these organisms the pigment occurs in a red ring around the nucleus, giving the appearance of an eye, from which the popular name, eye-spots, of *Euglena,* is derived. When these organisms turn green the chlorophyll develops first at the periphery of the red ring and gradually spreads inward. The red pigment does not always occur in *Euglena sanguinea,* however, and its absence seems to exert little if any effect on the normal development of the organisms. The eye-spots occur chiefly in spring and autumn, or when the organisms are in a dry state or exposed to bright sunlight. Cohn (1850) and Klebs (1883) regarded the red coloring matter as identical with that of the *Chlorophyceae, Haematococcus pluvialis,* the so-called haematochrome of Cohn. If this be the case the eye-spot pigment is a mixture of carotinoids, inasmuch as Zopf (1892a) has shown that haematochrome consists of carotin and

a red xanthophyll-like pigment whose exact relation to the ca
tinoids remains to be determined.

The red pigment of *Euglena sanguinea* was first isolated by
Wittich (1863) and later by Garcin (1889) and Kutscher (189
V. Wittich obtained microscopic, garnet colored octahedral crystals
concentrating the hot alcoholic extract or by adding alcohol to
concentrated ether solution of the pigment. The crystals were quic
bleached by chlorine and gave a blue color reaction with concentra
sulfuric acid. The crystals melted indefinitely between 70° and 1
C. They dissolved in hot alkali and the pigment could be recove
from this solution in amorphous form, but without loss of other pr
erties, by addition of acid. Neither Garcin nor Kutscher was a
to extract the pigment from *Euglena* cultures with cold alcohol,
the former obtained orange-red extracts with chloroform, follow
alcohol treatment, and the latter with boiling absolute alcohol. T
pigment extracted by Garcin showed no absorption bands, but d
solved in concentrated sulfuric acid with a blue color. Garcin p
posed the name rufin for the pigment. Kutscher's absolute alco
extracts deposited garnet colored crystals on concentration. T
pigment as described further by Kutscher does not seem to be a ca
tinoid because the recrystallized substance melted at 105° C. a
exhibited no absorption bands. The crystalline pigment, as well
its alcoholic solution, turned blue on addition of dilute (50 per ce
sulfuric or nitric acids, but alkalies had no effect.

Besides these more critical studies Krukenberg (1886) found th
saponified alcoholic extracts of *Euglena* would yield a greenish-yell
lipochrome to petroleum-ether or ether in addition to the red pigm
which acetic ether only would extract from the soap. The red p
ment, according to Krukenberg, showed one absorption band, wh
is contrary to the statement of the other investigators. The greenis
yellow pigment may have been a true carotinoid inasmuch as e
dence of carotinoids in *Euglena* was obtained by van Wisselin
(1915) who secured a positive Molisch carotinoid test on the e
spots. The chief pigment present, namely, the red one, does n
however, appear to be identical with any of the known carotinoi
but resembles more nearly the red carotinins of Zopf.

The Myxophyceæ (*Cyanophyceæ*). These constitute a large cl
of unicellular or filamentous algæ without a true nucleus, wh
inhabit both fresh and salt water and are also found in damp so
or on damp rocks and tree-trunks, forming dark blue-green patch

The algae frequently live in symbiosis with fungi or other plants. Their characteristic color gives them their common name, the blue-green algæ. They are among the lowest forms of plant life which are known.

The pigments of the blue-green algae have attracted the attention of a number of investigators beginning with Nägeli (1849) who expressed the belief that these organisms contain a special pigment, which he called phycochrome, present in two modifications, a blue-green phycocyan, and an orange phycoxanthin. It was thus that the latter name, later applied to the pigment of the *Phaeophyceæ,* had its origin. The various species of blue-green algæ which have been examined for carotinoid pigments are given in Table 11.

TABLE 11. MYXOPHYCEAE FOUND TO CONTAIN CAROTINOIDS

Order *Rivulariac*[illegible]ms).
 Calothrix [illegible] and Millardet, 1868.
 Rivularia [illegible], 1902.
Order *Scytonemaceæ.*
 Tolypothrix species—Kohl, 1902.
Order *Nostocacea.*
 Nostoc species—Kraus and Millardet, 1868; van Wisselingh, 1915.
 Nodularia—van Wisselingh, 1915.
 Anabaena flos aquæ Bub.—Tammes, 1900; van Wisselingh, 1915.
Order *Oscillatoriaceæ.*
 Oscillatoria—Sorby, 1873; Reinke, 1876; Monteverde, 1893.
 Oscillatoria (Oscillaria) limosa—Kraus and Millardet, 1868; Kraus, 1872.
 Oscillatoria [illegible] 1896.
 Oscillatoria [illegible], 1900; Kohl, 1902.
 Phormidium vulgare—Nebelung, 1878; Kohl, 1902.
Order *Chroococcaceæ* (lowest forms).
 Microcystis (Polycystis) flos aquæ Wittr. (fresh water)—Zopf, 1900.

A survey of the observations of the various investigators shows conclusively that carotinoids are normal constituents of the *Myxophyceae.* This has been demonstrated microchemically by Molisch (1896), Tammes (1900), Kohl (1902) and van Wisselingh (1915). Spectroscopic studies were made by Reinke (1876) and Nebelung (1878) on different species, and while the results indicate carotinoids, the solutions examined were not free from other pigments.

The evidence regarding the character of the carotinoids present is less conclusive. Kraus and Millardet (1868) found that alcoholic extracts of *Oscillatoria, Nostoc* and *Calothrix* species responded to the alcohol-benzene separation, leaving a yellow pigment behind in the alcohol. Nägeli's designation, phycoxanthin, was adopted for the pigment remaining in the alcohol. Kraus (1872), however, later recognized that more than one pigment was probably present in these

algæ, and expressed the belief that the pure xanthophyll-like phycoxanthin in *Oscillatoria* is closely related to, but not identical with, the xanthophyll of higher green plants.

Sorby (1873) differentiated between phycoxanthin, fucoxanthin and "orange xanthophyll" (carotin) in *Oscillatoria.* Sorby's phycoxanthin is not identical with the so-called phycoxanthin of brown algæ, the pigment now known as fucoxanthin. As described by Sorby phycoxanthin is practically non-extractable from alcohol by carbon disulfide, but when dissolved in the latter solvent gives red solutions, which are still pink in great dilution, the absorption bands in this solvent being shifted towards the red end of the spectrum to even a greater extent than those of carotin. The presence of such a pigment in *Oscillatoria* has not been reported by others, and its relation to the carotinoids remains to be determined. Sorby's fucoxanthin is identical with the fucoxanthin of the brown algae, whose chemical properties have been described. No other investigator has reported the presence of this pigment in the blue-green algæ. If Sorby's observations can be substantiated it will show that this pigment is much more universally distributed among the algae than has been heretofore regarded.

Sorby also reported observations regarding the distribution of phycoxanthin, "orange xanthophyll" and fucoxanthin in *Oscillatoria* with different exposures of the organisms to light, finding that the more intense the light during growth the more phycoxanthin and "orange xanthophyll" (carotin) they contain and the less fucoxanthin.

Further evidence regarding the presence of carotin in the blue-green algæ was furnished by Monteverde (1893), who demonstrated carotin in *Oscillatoria* by the Kraus method. The question of the character of the pigments remaining in the alcohol following the separation between this solvent and petroleum ether was left open by Monteverde.

That even the lowest forms are abundantly pigmented by carotin has been shown by Zopf (1900) who has described the ease with which carotin crystals can be obtained from *Microcystis* (*Polycystis*) *flos aquæ* Wittr. It is doubtful, however, whether Zopf is justified in regarding the carotin as a special pigment, and ascribing to it the name polycystin.

Carotinoids in the Fungi

The brilliancy of color which characterizes practically all classes of fungi is a fact which is familiar even to the layman in the fields of botany and biology. Yellow, orange and red colors are by no means the least conspicuous among these plants, and may in many cases be regarded as the predominating ones. This fact, together with the absence of chlorophyll from this form of plant life, is what gives prominence to a consideration of the relation of the pigments involved to pigments of similar color, namely, carotinoids, produced in the chlorophyllous plants. As has been already pointed out, however, yellow, orange and red colors in fungi appear to be more frequently non-carotinoid in nature than possessing the characteristics of the chromolipoids. Zopf (1890) mentions a number of instances where this is the case. Nevertheless, carotinoids do occur among the fungi, especially among the higher forms, and the evidence for this conclusion will now be presented. It will be apparent, however, that specific evidence is almost completely lacking as to the kinds of carotinoids involved. The plan of presentation will be similar to that followed in the case of the algae.

The Basidiomycetes. The various fungi which comprise this group include the numerous species of mushrooms, toadstools and bracket fungi (included together under the *Hymenomycetes*), the puff-balls (*Gasteromycetes*) known to every school child, the rusts (*Uredineae*), and the smuts (*Ustilagineæ*). Yellow colors do not especially characterize the *Gasteromycetes,* and so far as the author is aware carotinoids have not been demonstrated in any of the members of this family. The same statement likewise holds true for the smuts. Yellow to orange-red tints are very common, however, among the *Hymenomycetes,* and the *Uridineae* take their common name (rusts) from the predominating color of their spores.

The species of *Basidiomycetes* which may be regarded as owing their color to carotinoids or related pigments are collected in Table 12. It is not to be considered that this short list comprises all the species which probably contain carotinoids, but merely that proof has been furnished for those mentioned. For example, the common mushroom *Clavaria fusiformis* (Golden Spindle) may owe its color in part to carotinoids although Sorby (1873) in his study of the pigments of many classes of plants speaks of this fungus only as a source of

"lichnoxanthin," whose exact relation to known pigments is as yet obscure.

All of the *Hymenomycetes* which are known to contain carotinoids are bracket fungi which grow on decaying wood or among fallen leaves. Whether these fungi derive their carotinoids from the hosts upon which they grow or synthesize their own pigments remains to be determined.

Calocera viscosa is a very sticky fungus of a beautiful orange color which is found abundantly on rotten tree stumps, especially fir, in the autumn. It grows one to three inches high. *C. cornea* is not so highly colored and grows in spikes one-fourth to two-thirds inches high on dead wood. *Dacromyces stillatus* forms deep orange colored spots on pine and other decaying wood. *Ditiola radicata* produces a golden-yellow hymenium two to three inches across on rotten wood, and among fallen pine leaves, etc.

TABLE 12. BASIDIOMYCETES FOUND TO CONTAIN CAROTINOIDS

Hymenomycetes.
- *Calocera viscosa*—Zopf, 1889c; van Wisselingh, 1915.
- *Calocera cornea*—van Wisselingh, 1915.
- *Calocera palmata*—van Wisselingh, 1915.
- *Dacryomyces stillatus*—Zopf, 1889c.
- *Ditiola radicata*—Zopf, 1893a.

Uredineæ (rusts).
- [illegible] *juniperinum*—Bachmann, 1886.
- [illegible] *capreæ*—Bachmann, 1886.
- *Puccinia coronata*—Bachmann, 1886.
- *Triphragmium Ulmariæ*—Bachmann, 1886.
- *Uromyces alchemillæ*—Bachmann, 1886.
- *Coleosp*[illegible] S[illegible]auss—Muller, 1885; Bertrand and Poirault, 1892.
- *Uredo* [illegible] and Poirault, 1892.
- *Melampsora accidioides* D. C.—Müller, 1886; Bertrand and Poirault, 1892.
- *Phragmidium violaceum*—Müller, 1886.
- *Accidia, Promycelia* and *Sporidia Spores*—Kohl, 1902.

The evidence that these fungi owe their color to carotinoids was first furnished by Zopf (1889c) who found that the chromolipoid when isolated not only responded to the blue color reaction with concentrated sulfuric acid, which Zopf called the lipocyan reaction, but could be made to produce blue microscopic crystals under the influence of this reagent. Zopf described in detail the method for the formation of the blue "lipocyan" crystals for the pigments of these fungi and also for other lipochrome containing materials. More conclusive evidence that these fungi owe their color to carotinoids was furnished by Zopf (1893a) who isolated a pigment showing the spectroscopic absorption bands of carotin from *Ditiola radicata*, and

more recently by van Wisselingh (1915) who was able to secure crystals of pigment by the Molisch microchemical test. The latter investigator states that about twenty-five fungi which he examined by this method failed to respond to the test, but mentions specifically only those which responded. Special attention was given to the crystals produced in the case of *Calocera viscosa* and *Dacryomyces stillatus*. From the former a heavy precipitation of orange colored crystals was secured from a section between the hypens. These gave all the carotinoid color reactions, and the crystals dissolved slowly in phenol-glycerin, indicating a xanthophyll-like pigment. *C. cornea* and *C. palmata* gave like results. In the case of *Dacryomyces stillatus* crystals of a similar color were secured, as well as red colored crystals and orange-yellow aggregates, suggesting the possibility of several carotinoids being present.

Particularly interesting is the abundant evidence that the coloring matter of the rust fungi is carotinoid in nature. Bachmann (1886) first called attention to the fact that the rusts from several different hosts owe their color to orange or yellow oil globules containing unsaponifiable chromolipoids. The pigment was isolated by cutting out the rust spots, extracting them with ether or hot alcohol, saponifying the extract, and extracting the soap with petroleum ether. The positions of the spectroscopic absorption bands of the extracts thus obtained correspond closely with those of carotin. The residues from the extracts gave the usual color reactions with concentrated sulfuric acid and iodine. Müller (1886) observed that red pigment crystals appeared in the spores of certain rusts when placed in glycerin. Zopf (1890) held that these were due to a pigment other than lipochrome, but that the fungi contain the lipochrome as well as the special red pigment. Bertrand and Poirault (1892), however, who observed the same phenomenon in the rusts examined by Müller, as well as other species, regarded the red crystals to be due to cholesterol colored by carotin, inasmuch as identical crystals are formed when the pollen grains of *Verbascum thapsiforme* L. (mullein) are mounted in glycerin. In view of the observations of Bachmann pointing conclusively to the presence of carotinoids in the rusts, it seems likely that pigments of this type are involved in the crystals observed by Müller and by Bertrand and Poirault. It should be noted, however, that the uredo spores of these fungi, which is the chief pigmented stage in their development, are not colored alike for the various species, the color varying from yellow to reddish orange. The winter

stage, or teleutospores, is usually black, but in the case of one of the species mentioned in Table 12, namely, *Uredo euphrasix* Schum., this stage is red. Other stages in the life cycle of the rusts appear to contain carotinoids also, since Kohl (1902) reports a positive Molisch test on promycelia, sporidia and æcidia of the fungi, the consecutive stages in the germination of the teleutospores to the uredospore stage.

The Ascomycetes. These fungi, which are commonly known as the cup or sac fungi because of their shape, are frequently brilliantly colored with yellow, orange or reddish pigments. The coloring matter responsible for these tints has naturally attracted the attention of a few of the pigment workers, notably Zopf, to whom we owe much of our knowledge regarding the fungi pigments. The species of *Ascomycetes* whose pigmentation may with some assurance be regarded as due largely to carotinoids are mentioned in Table 13. No doubt others could be added to the list.

TABLE 13. ASCOMYCETES FOUND TO CONTAIN CAROTINOIDS

Discomycetes.
- *Peziza aurantia*—Zopf, 1892b.
- *Peziza bicolor* Büll.—Bachmann, 1886.
- *Peziza scutellata* L.—Bachmann, 1886.
- *Leotia lubrica*—Zopf, 1890, 1892b; Kohl, 1902.
- *Ascobolus species*—Zopf, 1889c, 1892b.
- *Spathularia flavida* Pers.—Zopf, 1892b; Kohl, 1902.

Pyrenomycetes.
- *Polystigma rubrum*—Zopf, 1893a.
- *Polystigma ochraceum* Wahlenberg (= *P. fulvum* D. C.)—Zopf, 1893a.
- *Spaerostilbe coccaphila*—van Wisselingh, 1915.
- *Nectria cinnabarina*—Bachmann, 1886; Zopf, 1893a; Kohl, 1902; van Wisselingh, 1915.

The *Peziza* genera of the *Discomycetes* contains several species with especially bright color. *Peziza aurantia,* which is sometimes called "orange-peel Elf-cup" takes the form of a shallow, irregular shaped cup, one to three inches in diameter, and resembles closely a piece of inverted orange peel. The outside of the fungus is pale orange but the interior is a brilliant orange or orange red. It is frequently found on the flat ground in autumn. *Peziza bicolor* Büll. forms a yellow to deep orange-red disc on dead branches of oak, hazel and hawthorn trees, and *P. scutellata* L. a deep carmine colored disc on rotten tree stumps. Sorby (1873) examined the pigment of the first mentioned species but called the pigment peziza xanthin and did not identify it with the "xanthophylls" of the higher plants or the algae. Bachmann (1886) first showed the presence of chromolipoids in the *Peziza* fungi when he recovered unsaponifiable pigment from them showing the

spectroscopic absorption bands and color reactions of the lipochromes from higher plants. This was confirmed by Zopf (1892b) for *Peziza aurantia,* who at the same time reported lipochromes in two other *Discomycetes,* namely, *Leotia lubrica* and *Spathularia flavida* Pers., but was unable to find lipochrome in *Bulgaria inquinans* (= *polymorpha*). The presence of carotinoids in the two first mentioned was later confirmed by Kohl (1902) using the Molisch test. *Leotia lubrica,* however, owes its color in part to a green colored pigment as well as to chromolipoid (Zopf, 1890). It is of interest to note also that Zopf (1889c, 1892b) reported that carotinoid-like pigments could be isolated from various species of *Ascobolus,* which flourish on the feces of animals.

Among the *Pyrenomycetes* Bachmann (1886) first reported unsaponifiable lipochrome in *Nectria cinnabarina* (Tode) Fries., which was later studied in detail together with the pigments of *Polystigma rubrum* Pers. and *P. ochraceum* (= *P. fulvum* D. C.) by Zopf (1890, 1893a). The former is a cushion shaped, red fungus found on the dead branches of deciduous trees, while *Polystigma* attack the foliage of plum trees, forming red or red-brown spots on the leaves.

The lipochrome which Bachmann isolated from *Nectria cinnabarina* corresponded in spectroscopic bands with xanthophyll. A red resin was also reported in this fungus. Zopf used the conidial layer of the fungus obtained from *Aesculus Hippocastanum* for his study. The presence of a two-banded "carotin" was confirmed and the red resin of Bachmann was found to conform to a number of other "carotinins" studied by this investigator (e.g., the red pigment of *Haematococcus pluvialis* already discussed) in that it readily formed compounds with sodium and barium. The sodium salt was practically insoluble in alcohol and ether, but soluble in chloroform, benzene and carbon disulfide, and the barium salt was insoluble in all these solvents. The ethereal solution of the base-free pigment showed two bands at 512-490$\mu\mu$ and 481-464$\mu\mu$, the solution in carbon disulfide showing three bands at 575-553$\mu\mu$, 530-508$\mu\mu$ and 494-482$\mu\mu$. The relation of this pigment to the carotinoids remains to be determined. It was either this pigment or the yellow "carotin" which responded to the Molisch test in the hands of Kohl (1902) and van Wisselingh (1915). Zopf called the red pigment nectriin or nectria red.

Zopf found two pigments in *Polystigma rubrum* which were very similar to those in *N. cinnabarina,* the absorption spectra of the "carotin" indicating identity with the carotin of carrots, the red pig-

ment, which appears to be the chief one present, differing from nectriin in the position and number of the absorption bands (polystigmin, as Zopf calls it, showing only two even in carbon disulfide), and also in that the barium compound is soluble in ether, chloroform, carbon disulfide and alcohol. Zopf's examination of *Polystigma ochraceum,* which has more of a yellow than a red color, showed an abundance of yellow "carotin," which was regarded as produced in the fungus cells. No red pigment was found, but the fungus was not decolorized after the extraction of the carotinoid, but was left a reddish-brown color which could be extracted by dilute ammonium hydroxide.

Van Wisselingh (1915) made a special examination of the microchemical crystals formed in *Spaerostilbe coccaphila,* a red fungus found on fallen trees. The fungus itself contains red, fat-like globules. Violet-red crystals were produced in the Molisch test, which gave the carotinoid color reactions and dissolved readily in the phenol-glycerin reagent which appears to be specific for xanthophyll.

The Phycomycetes. This class of fungi includes the molds, the mildews and the yeasts and thus contains many species of plants of great importance. One does not ordinarily associate carotinoid colors with these fungi, and the presence of such pigments does not, in fact, appear to be common. Carotinoids have been demonstrated to be present, however, in several instances.

Zopf (1892b) was able to extract a carotinoid from three species of *Pilobolus,* namely, *P. crystallinus, P. Kleinii* and *P. Oedipus,* which gave the lipocyan reaction, the lipochrome reaction with iodine, and also showed absorption bands in petroleum ether at 484-469μμ and 452-439μμ, which correspond closely with xanthophyll. The first two species flourish on fresh horse dung, the last on dung or rotting algae. Zopf also stated (1892b) that *Pleotrachelus fulgens,* a reddish-brown species of another order, is a carotin (oid) former, but the evidence for this was not presented.

Kohl (1902) confirmed the presence of carotinoids in *Philobolus* species using the Molisch test, and also showed the same pigments to be present in *Mucor* species and in *Chytridium.*

Van Wisselingh (1915) included *Mucor flavus* Bainer in his microchemical studies. Orange-yellow crystals were secured by the Molisch method, which gave the carotinoid color reactions with nitric acid and with bromine.

The Myxomycetes. These fungi form a distinct, independent group of plants, commonly known as slime molds, and were formerly classi-

fied in the animal kingdom under the name *Mycetozoa.* The plants are naked masses of protoplasm, called plasmodia, which exhibit many beautiful colors as shown in Lister's well-known work on the slime molds. Carotinoids are probably rare in this group, although this statement may be hasty inasmuch as very few species have been examined. Carotinoids do not appear to be present in *Arcyria punicea* Pers. and *Ar. nutans* Büll. or in *Aethalium septicum* Fr., a species of *Fuligo Septica*—the well-known "Flowers of Tan"—according to the observations of Schroeter (1875), Zopf (1892b) and Bachmann (1886). Carotinoids do appear to be present, however, in *Stemonitis ferruginea, Stemonitis fusca, Lycogala epidendron* and *Lycogala flavofuscum,* judging from the observations of Zopf (1889b) who made a special study of the possible presence of lipochromes in *Myxomycetes.* In no case was lipochrome found to be the only pigment present, although absolute alcohol was found to extract completely the color from the carrot-red plasmodia and fruits of *L. epidendron.* In each case an unsaponifiable lipochrome was isolated showing the color reaction with concentrated sulfuric acid. The measurements of the position of the absorption bands of the lipochrome of each species as reported by Zopf indicate a xanthophyll-like pigment in the case of *Stemonitis,* but carotin in the case of *Lycogala.* These observations might well be amplified by others, carried out in the light of our present knowledge of the carotinoids.

The Imperfect Fungi. There is evidence that carotinoids are present in a few species of this large group of fungi whose exact classification has not yet been determined.

Zopf (1889c) states that the pigment which can be extracted with fat solvents from *Cephalothecium* gives the blue lipocyan crystals with sulfuric acid. Several of the fungi which gave positive evidence of carotinoids microchemically in van Wisselingh's study (1915) belong in the group of imperfects. For example, *Monilia sitophila* (Mont.) Dacc. gave red crystals in the Molisch test; *Aspergillus giganteus,* which has an orange-yellow mycelium, gave a positive test; *Torula rubra* also gave the reaction, but *Torula cinnabarina* failed to do so, although a color reaction was secured using $SbCl_3$.

Carotinoids in Bacteria

The importance of bacteria as a means of determining some of the true functions of carotinoids in plants, or at least of fixing the con-

ditions under which they develop, has already been pointed out. The work upon which our present knowledge of carotinoids in bacteria is based will now be reviewed. Bacteria are at present classified as *Schizomycetes*, and are best considered as algae. Their morphology and reproduction most nearly resemble the *Cyanophyceae*. In fact, bacteria are considered by some as having "degenerated" from the blue-green algae. They do not, however, contain chlorophyll, and it is this fact, especially, which enhances the interest in the possibility of carotinoids being normal constituents of these organisms.

As in the case of non-chlorophyll bearing fungi, it is not to be assumed that all yellow, orange and red tinted bacterial colonies owe their color to carotinoids. The pigments of *B. prodigiosus* and *B. xanthinum* Ehren. first described by Schroeter (1875) are obviously not carotinoids, although color alone would suggest that this is the case. Griffiths (1892) ascribes the formula $C_{38}H_{56}NO_5$ to the red pigment of *B. prodigiosus*, but the empirical relation between the carbon and hydrogen suggests, rather than negatives a relation of the pigment to carotin. Schroeter described the change of color of the colonies of this bacteria from red to orange to yellow and ascribed it to the formation of an alkaline substance in the course of the growth of the bacteria. This variation in color of *B. prodigiosus* is probably well known to bacteriologists and might be thought to be due either to a variation in concentration of the same pigment or to the presence of distinct yellow (possibly carotinoid) and red pigments, the latter, when present, masking the former. Schroeter supported his explanation of the change in color, however, by showing that the orange-red alcoholic extract of the bacteria turns red with acid and yellow with alkali.

Aside from the brief observation of Schrötter (1895) that the pigments of *Sarcina aurantiaca* and *M.* (*Staph.*) *pyrogenes aureus* show the solubility properties and color reaction (with H_2SO_4) of "lipoxanthin" (carotinoid) our knowledge regarding carotinoid producing species of bacteria is due apparently solely to Zopf (1889, a, c; 1891; 1892b) who has described the chromolipoids in eight species of bacteria. The descriptions as given by Zopf point with certainty to carotinoids in the case of four species only, namely, *B. egregium*, *B. Chrysogloia*, *M.* (*Staph.*) *aureus* and *Sphaerotilus roseus*. The first three of these bacteria form yellow colonies, but the last mentioned species is red. The evidence for carotinoids is as follows:

B. egregium. Forms intensely yellow colonies on gelatin or beef-

extract agar (b.e. 2.3 per cent, agar one per cent). Colonies when transferred to porcelain plate give blue color with concentrated H_2SO_4 and HNO_3, and blue microscopic crystals with former (lipocyan reaction, Zopf). Pigment is slowly extracted by warm absolute alcohol and when thus extracted is soluble in alcohol, ether, chloroform, methyl alcohol, benzene and petroleum ether. The alcoholic solutions show two absorption bands, one covering the F line, the other between F and G. The pigment is not saponifiable. It develops in the dark as well as in the light.

B. Chrysogloia. The yellow pigment produced corresponds exactly in properties with that of *B. egregium.*

M. (*Staph.*) *aureus.* The yellow pigment shows the same properties described for the above mentioned bacteria, according to Zopf.

Spaerotilus roseus. A red bacteria giving a yellow to yellowish-red alcoholic extract. Strips of filter paper immersed at one end in the extract showed in time three zones, a wide red zone over which was a narrow yellow zone and over this a very narrow brownish zone. The yellow pigment was soluble in water and the red one in alcohol, ether, chloroform, ligroin, petroleum ether, benzene and carbon disulfide. After saponification and extraction with petroleum ether the pigment showed all the properties of "eucarotin," the absorption bands in alcohol lying at 492-474$\mu\mu$ and 456-442$\mu\mu$. The properties described are strongly indicative of carotin.

There is much less certainty regarding the character of the pigments in the other species of bacteria examined by Zopf, although the pigment is ascribed by Zopf to "lipochrome." The red color of *M.* (*Staph.*) *apatelus* and *M.* (*Staph.*) *superbus* is stated (1889c) to be a red lipochrome which gives the microscopic blue lipocyan crystals with concentrated H_2SO_4. The red pigment of *M.* (*Staph.*) *rhodochrous* and *M.* (*Staph.*) *Erythromyxa* gives the same reaction. Old colonies of these two bacteria show scarlet or blood-red crystal aggregates under the microscope (dark field), according to Zopf (1891). These crystals are soluble in alcohol, ether, chloroform, petroleum ether, benzene and carbon disulfide and these solutions are characterized by showing only one wide absorption band in the spectroscope at F. The pigment is not saponifiable. It is not clear whether this pigment is one of the known carotinoids or is to be classified with the red "carotinins," which have been repeatedly mentioned, and the determination of whose relation to the carotinoids is greatly to be desired. Overbeck (1891), who has studied the physiology of pig-

ment production in the two last mentioned species of bacteria, states that *M. Erythromyxa* produces a yellow water-soluble pigment in addition to the red lipochrome.

Summary

Our knowledge is practically complete regarding the character and distribution of the carotinoids among certain classes of algae, particularly the brown and red sea-weeds.

Fresh brown sea-weeds owe their olive-brown tint to the special algae carotinoid, fucoxanthin, discovered by Rosanoff (1867) and Millardet (1869), and finally classified definitely as a carotinoid by Willstätter and Page (1914). The relation of this pigment to carotin is shown by the empirical formula $C_{40}H_{56}O_6$. The characteristic properties of the pigment are described in detail in the text.

Brown sea-weeds also contain carotin and xanthophyll. The exact relation of this xanthophyll to the xanthophylls of higher plants has not been definitely settled.

Dried brown sea-weeds owe their color to phycophäin, a post-mortal oxidation product of colorless chromogens present in the fresh plants. The carotinoids are still present, but the phycophäin interferes greatly with their isolation and study.

The principal pigment of red sea-weeds is phycoerythrin, which is not a carotinoid. Carotin and xanthophyll, however, are present in these plants. There are some indications that the xanthophyll is the xanthophyll β which characterizes higher plants. There is a possibility, also, that fucoxanthin is present in the red algae.

Carotinoids are present in the stone worts, but nothing is known of their nature.

Carotin and xanthophyll are present in the green algæ, the amount of each present in certain species having been determined by Willstätter and Page. The red pigment of the so-called blood algae classified among this family, appears to be related to the carotinoids, but its exact relation remains to be determined.

Carotin appears to be the principal carotinoid present in the diatoms. There is a possibility, also, that xanthophylls and fucoxanthin are present, a phase of the pigmentation of the siliceous algæ which deserves further study.

Our knowledge is indefinite regarding the carotinoids occurring in the *Peridiniales,* although the indications are that a xanthophyll-like

pigment, possibly fucoxanthin, predominates among the chromolipoids present. Brownish-red and yellow water-soluble non-carotinoids are the chief cause of the color of these plants.

Carotinoids are unquestionably present in the flagellates, although their exact nature remains to be determined. The red pigment which characterizes the so-called eye-spots of *Euglena* species does not appear to be identical with any of the known carotinoids, but resembles the red carotinins, the determination of whose relation to the carotinoids is greatly to be desired.

Carotinoids are normal constituents of the blue-green algae. The facts which are known point to the presence of carotin and fucoxanthin in these plants. Another pigment is present in certain species, which is non-carotinoid in nature but which resembles carotin in having a yellow color in alcohol and a red color in carbon disulfide. Its exact nature is not known.

Carotinoid colors are more common among the fungi than among the algæ but the color in many cases appears to be due to other pigments. In fact, many fungi seem to be entirely devoid of carotinoid pigments.

Among the *Basidiomycetes*, a few species in the mushroom family apparently owe their color to carotinoids. The striking examples of carotinoid pigmentation, however, are the rusts, whose yellow and red colors are due to carotin or a very closely related pigment. It is not known whether other carotinoids are involved.

Several of the brilliantly colored cup fungi owe their color to carotinoids. The exact nature of these has not been determined in the case of the *Discomycetes*, but in the case of certain *Pyrenomycetes* carotin is undoubtedly concerned, as well as red carotinoid-like pigments which require further study.

Carotinoids have been identified in a few molds and yeasts but their nature is unknown.

There is considerable uncertainty regarding the exact relation to the carotinoids of certain yellow pigments characterizing the slime molds, but xanthophyll or carotin-like pigments are indicated in the case of certain species.

Carotinoids are formed by several species of bacteria. Carotin appears to be the principal pigment concerned in the case of *B. egregium* and *Spaerotilus roseus*. The exact nature of the pigment has not been determined in the case of the other species in which carotinoids have been determined to be present.

Carotinoid-forming bacteria afford an excellent opportunity for fixing the conditions under which these pigments develop and thus throwing some light on the true functions of carotinoids in plants. The growth of these plants is subject to very exact laboratory control and their pigmentation is not complicated by the formation of chlorophyll.

Chapter IV

Carotinoids in the Vertebrates

It is by no means a new idea that certain pigments, widely distributed among animals, resemble closely in their chemical and physical properties, as well as in color, the pigments of the vegetable kingdom which were considered in the preceding chapters. This point was brought out in Chapter I. The demonstration of a general biological relationship of these animal pigments to the plant carotinoids is, however, comparatively recent. It is because of this relationship that one is justified in considering the carotinoids of plants and animals in one treatise. The development of this idea and the experimental justification for it are reserved for presentation in a later chapter. It is accordingly necessary to anticipate this discussion at this point and to review the evidence for the distribution of the carotinoids among animals without having first justified the basis for this distribution. The reader is therefore asked to assume for the moment that the yellow to orange-red animal pigments which have been most commonly called lipochromes are in all probability true or modified plant carotinoids. For certain of the higher animals proof has been furnished that their lipochromes are true carotinoids, but this knowledge does not as yet extend very far down the scale of animals. However, the thread is picked up again for certain of the lower animals so that it does not require a difficult stretch of imagination to fill in the gap, wide as it is indeed admitted to be.

Carotinoids in Mammals

Corpus luteum. Bearing in mind that carotin was the first vegetable chromolipoid discovered, it is an interesting fact that the first mammalian chromolipoid to be isolated in crystalline form likewise eventually proved to be carotin. The pigment referred to is that of the corpus luteum of the cow, first described by Piccolo and Lieben (1866) and a little later, apparently independently, by Holm (1867). As already mentioned in Chapter I, the former called the pigment

luteohämatoidin or haemolutein, while Holm called it hämatoidin. Of the two papers mentioned that of Holm, only, has been accessible to the writer. It is gratifying to note how accurately Holm described the crystalline form, the color of the crystals, both alone and when dissolved in various solvents, and the characteristic blue color reaction with nitric acid, all of which later helped to identify the pigment as carotin. The close relationship of the corpus luteum pigment to other yellow pigments in plants and animals was first recognized by Thudichum (1869), but his supposition that most of these pigments were identical has since proved to be without foundation, although his ideas in this respect were in part correct.

Capranica (1877) likewise isolated the corpus luteum pigment from cow's ovaries and obtained it in crystalline form. The general properties (color reactions, spectroscopic absorption bands and solubility) corresponded so closely with those of the pigment of the yolk of eggs (hen) and the pigment in the retina of the eyes, as examined by this investigator, that he regarded the three pigments as identical. This conclusion led him to regard this pigment as one of the most important substances in living matter. The following quotation from Capranica's paper is, to say the least, the most enthusiastic conception of the part which carotinoids play in animal life, which the writer has encountered. "Diese Substanz muss demgemäss als eine der phylogenetisch ältesten chemischen Verbindungen des thierischen Körpers angesehen werden. Wir dürfen annehmen, dass schon in den ersten Regungen der organischen Materie das lichtempfindliche Molecul des Lutein vorhanden sein. Die erste Entstehung dieses Molecüls, kann man sich denken, war das *'Fiat Lux.'* Mit ihr begann zwischen Sonne und organischer Materie jene empfindende Verbindung, als deren letzte und höchste Frucht wir des Menschen sonnenhaftes Auge anstaunen."

The full significance of Capranica's contributions, however, was not appreciated by him or by subsequent investigators of animal chromolipoids. He observed, among other things, that petroleum ether and carbon disulfide, respectively, would quantitatively remove the corpus luteum pigment from its alcoholic solution. The development of the technic for separating carotin from other pigments by this method is a comparatively recent achievement, as shown in the preceding chapters. If Capranica had thought to apply this test to the egg yolk pigment which he had under investigation he would have discovered a difference which may have led to a much earlier discovery

of the true relationship of the corpus luteum and egg yolk pigments to each other and to other similar pigments in plants and animals. At any rate, much of the subsequent confusion of different pigments might, perhaps, have been avoided.

Kühne (1878), however, was forced to conclude that the corpus luteum and egg yolk pigments were not identical, after examining carefully their spectroscopic absorption properties. No further study appears to have been made of the corpus luteum pigment until Escher (1913) definitely established its identity with carotin.

Before referring to other mammalian carotinoids it may be well to point out that we have definite proof that carotin is the corpus luteum pigment only in the case of cows and sheep, from which Escher obtained his material for study. Pigmented tissue appears on the human ovary, also, but there is no evidence that the pigment is exclusively carotin. On the contrary the inference which may be drawn from observations regarding the character of the chromolipoids in other parts of the human body is that both carotin and xanthophylls probably appear in the human corpus luteum. Still less is known regarding the pigment in the corpus luteum of other mammals. In the horse it is probably carotin, since this pigment appears in the blood of that animal. Carotinoids are not present at all in the so-called yellow bodies on the ovaries of swine, as pointed out by van den Bergh, Muller and Broekmeyer (1920). The writer[1] succeeded in extracting a small amount of yellow coloring matter from swine ovaries when a sufficient number were extracted, but all attempts to identify the pigment as carotinoid resulted in failure.

Blood serum. Although the carotinoid of the corpus luteum of the cow was the first mammalian chromolipoid to be isolated in crystalline form, the coloring matter in the blood serum of cattle was probably the first to attract attention. Krukenberg (1885a), who deserves credit for the first extensive study of the pigment, mentions the much earlier attempts of Samson (1835), Denis (1838) and Schmidt (1865) to determine its nature. It is true that Thudichum (1869) stated that the yellow pigment of blood serum belonged to his group of luteins, but he did not trouble to mention the animals in which he had found it, or how he had isolated the pigment. As a matter of fact Krukenberg (1885a) found it to be rather difficult to separate the pigment of cattle serum from the other blood constituents; direct extraction with all the known fat solvents failed completely, and

[1] Unpublished observations.

success was attained only by repeated extractions of the serum with amyl alcohol. The writer's study of blood serum pigments of cattle has shown that this difficulty is readily explained. When the chromolipoid present is carotin, the pigment is physico-chemically attached to serum-albumin. Alcohols have a greater attraction than pigment for the colloidal protein and thus replace it. Fat solvents will then extract the pigment, petroleum ether being the best solvent to use.

Krukenberg's observations of the pigment isolated by him from ox serum were confined to solubility properties in the lipochrome solvents, the color reactions with concentrated H_2SO_4 and HNO_3, and the spectrum bands of the pigment. Positive identification as a lipochrome was secured in each case. Krukenberg was careful to recognize that pronounced spectroscopic differences among lipochromes indicated the existence of more than one individual in his lipochrome group. On these grounds he was led to conclude that the blood serum pigment of the ox is probably identical with the lutein of the corpus luteum, whose spectrum properties had been previously pictured by Kühne. The additional interesting observation was made that the fresh serum itself showed the spectrum bands, although shifted considerably towards the red end of the spectrum from their position in chloroform or ether. The writer was unable to verify this for a specimen of human blood serum which proved to be rich in carotin. Although Krukenberg made no attempt to identify the pigment with any of the vegetable lipochromes with which he was familiar, his graphic representation of the spectrum of the cattle serum pigment shows it to be identical with that of carotin. Krukenberg had no explanation to offer for the occurrence of the pigment in the blood. He was opposed to the view that it originated from hemoglobin, but nevertheless saw an analogy between the simultaneous occurrence of lipochrome with the respiratory pigment of both plants and animals. Van den Bergh and Snapper (1913) confirmed the general observations of Krukenberg regarding the properties of the pigment of cattle serum. In addition, they noted traces of bilirubin in the serum and proposed an interesting test for the presence of both lipochrome and bilirubin in blood serum based on their observation that the lipochrome of cattle serum is precipitated with the proteins when two volumes of 95 per cent alcohol are added to one volume of serum while bilirubin remains in the supernatant fluid when the precipitated proteins are centrifugalized. Definite identification of the lipochrome of cattle serum as carotin was made by Palmer and Eckles (1914c)

by applying the numerous macroscopic tests for this pigment evolved by the plant biochemists, particularly Willstätter and Mieg and Tswett. This fact has recently been confirmed by van den Bergh and Muller (1920) and by van den Bergh, Muller and Broekmeyer (1920). In addition, Palmer and Eckles found that xanthophylls could also be demonstrated in small amounts in well-colored serum if sufficient material (250-300 c.c.) was used. Neither type of carotinoid was present in the blood of a newborn calf.

After Hammarsten (1878) isolated crystalline bilirubin from horse serum, it was believed for many years that this pigment was the sole cause of the well-known golden yellow color of the serum of this mammal. Gallerani (1904), however, found a lipochrome-like pigment accompanying the bilirubin in horse serum, for which he proposed the name plasmachrome. Van den Bergh and Snapper (1913), also, found some lipochrome accompanying the bilirubin in horse serum. The carotinoid identity of this lipochrome was shown a little later by the writer (1916), using serum from a horse on bluegrass pasture (rich in carotinoids). Carotin only was found, adsorbed on the albumin, as in the case of cattle serum, although the quantity present in a unit volume was considerably less than was found in cattle serum under comparable feeding conditions. Van den Bergh and Muller and Broekmeyer (1920) have confirmed these findings, also, in so far as the character of the carotinoid and the amount present are concerned.

Since Thudichum's (1869) early observation it has been recognized that human blood serum may be colored by a lipochrome. Zoja (1904) found that bilirubin is not present except under pathological conditions. However, van den Bergh and Snapper (1913) state that the serum of normal persons always contains a certain amount of both lipochrome and bilirubin, sometimes one and sometimes the other being in excess. They observed, also, that the serum of diabetics may contain extraordinarily large amounts of lipochrome, an observation subsequently confirmed by Umber (1916), Bürger and Reinhart (1918, 1919), Salomon (1919), van den Bergh and Muller (1920), van den Bergh, Muller and Broekmeyer (1920), and by Head and Johnson (1921). Umber was able to shake the pigment out of the serum with ether alone. Bürger and Reinhart (1918) suggested that the serum pigment might be of exogenous origin and later (1919) presented quantitative data showing a rise in the pigmentation of the serum on a diet of green food. Salomon definitely identified as carotin the

pigment which he extracted from high colored human serum. Of interest is his observation that in this case direct extraction with ether took out very little pigment, it being necessary first to precipitate the proteins with alcohol and extract the precipitate with ether. Apparently this investigator regarded the presence of the carotin as fortuitous, for he mentions the difficulty in distinguishing the pigment from the normal lipochrome of the blood.

It is obvious that none of the workers mentioned in the preceding paragraph were familiar with the observations of the writer on the character and cause of the normal chromolipoid of cattle and horse serum. It remained for Hess and Myers (1919) to show the direct application of the writer's observations on animals to the variations in the pigmentation of human blood serum, by demonstrating marked variations in the carotin content of the blood serum of children with variations in the carotin content of their diet. These observations have been extended greatly by van den Bergh and Muller (1920) and van den Bergh, Muller and Broekmeyer (1920) who have shown that both carotin and xanthophylls play a part in causing the normal pigmentation of human blood serum, sometimes one and sometimes the other predominating, although carotin is usually in excess.

The writer has recently observed an interesting case of marked change in the character of the carotinoid in the blood serum of an adult. At the time of the first examination the serum was colored almost exclusively by carotin, which could not be shaken out of the blood with ether. At this time carrots played a large part in the diet. At the time of the second examination the pigment was readily extracted simply by shaking the serum with ether. The character of the diet was not ascertained in this case, although a similar pigment, readily extracted by ether, was found abundantly in the blood of two other persons on a diet rich in green foods (spinach and green string beans). By analogy with the writer's (1915) experiments with the pigment of fowl serum this pigment should have been xanthophyll. However, a phase test applied to the pigment in each case showed that it was almost quantitatively epiphasic between the petroleum ether and 80 per cent methyl alcohol. Inasmuch as this property is supposed to be distinctly characteristic of carotin, it appears that the character of the diet may influence the manner in which carotin is carried by human blood. In each case the serum extracts showed two-banded absorption spectra when using a spectroscope with narrow dispersion, but it was not possible to secure the measurements of

the bands when using an instrument of high dispersion equipped for measuring the wave-length positions of the bands.

A word may be said here regarding the state of carotinoids in blood. Van den Bergh and Muller (1920) assert that neither carotin nor xanthophylls can be shaken out of blood serum with ether. They believe that the pigments are always in colloidal solution in the plasma. The writer is in accord with this view in so far as carotin in ox and horse serum is concerned, and at times for human serum. It is believed, however, that in all probability a double colloidal phenomenon is involved in these cases, i.e., first, a colloidal adsorption of the carotin by albumin and second, a colloidal solution of this albumin in the plasma. As for xanthophyll in blood serum, the writer merely wishes to state that he has never failed to secure its direct extraction with ether when present in the serum of animals, and accordingly does not feel justified in believing that colloidal phenomena are involved in any way. The explanation for this difference offers an interesting problem in biochemistry.

Observations are very scanty on the pigment of the blood serum of other mammals. The writer (1916) examined the blood of each of three breeds of swine, representing the Duroc-Jersey, Poland China and Berkshire breeds at a time when they were on pasture, but failed to detect the presence of even traces of carotinoid or other chromolipoid-like pigment. In a similar manner the blood of each of five breeds of sheep, namely, Dorset, Hampshire, Merino, Shropshire and Southdown, showed the presence of only traces of chromolipoid, which appeared to be carotin, although the animals, like the swine, were receiving an abundance of carotinoid-rich pasture grass at the time. The blood of an Angora goat, under like feeding conditions, showed traces of carotinoid also. Van den Bergh, Muller and Broekmeyer (1920) likewise found no carotinoids in the blood serum of swine, guinea pigs or dogs, and traces only in the blood serum of cats. In the case of the latter animal, xanthophyll practically disappeared from the blood within a half hour after an intravenous injection of a colloidal solution of xanthophyll. It is stated that the pigment was found, however, in the liver.

Milk fat. Thudichum's classic paper included the pigment of butter fat among the "luteins." Blythe (1879), however, regarded the alcohol soluble lactochrome which he isolated from milk whey as the cause of the butter fat color, and Desmouliere and Gautrelet (1903) concluded, after isolating a urobilin-like pigment from milk,

that no lipochromes are present. On the other hand the fact that the pigment of butter fat appears in the unsaponifiable ether extractable material at once classifies it as a chromolipoid. Palmer and Eckles (1914a) were the first to make a critical examination of the pigment from the standpoint of the plant carotinoids, finding, as might be expected in the light of Escher's work on the corpus luteum pigment of the cow, that the pigment corresponds exactly in physical and chemical properties (spectroscopic, solubility and phase test) with carotin. In addition we found, when the phase test and a chromatographic analysis were applied, that small amounts of xanthophylls usually accompany the carotin. These were most evident in highly colored butter fat, a chromatogram in one case showing two and possibly three distinct adsorption zones of xanthophyll. The pigment in each of these zones showed the xanthophyll absorption bands and were hypophasic in the phase test between petroleum ether and 80 per cent alcohol.

The character of the carotinoids in the milk fat of other animals has not been determined. Palmer (1916) and Palmer and Kennedy (1921) have noted the presence of carotinoid in traces in the milk fat of sheep and goats without determining which kind of carotinoid is present. We have also noted a complete absence of carotinoids from the milk fat of albino rats and swine, even the fat of the colostrum milk of the latter.

The fat of human milk is always more or less pigmented, that of colostrum being especially highly pigmented. Palmer and Eckles (1914e) found both carotin and xanthophylls in about equal quantities, as judged from the color of the solutions obtained in the phase test when applied to the isolated pigment. Two samples of human milk were examined, from different individuals, one sample being colostrum. This result is to be expected in the light of what has been found subsequently regarding the presence of both types of carotinoids in human blood.

Adipose tissue. The adipose tissue of cattle, horses and man is characterized by varying amount of pigment, which at times attains a high concentration in the horse, in certain breeds of cattle, such as the Jersey and Guernsey dairy breeds, and at times in man. The adipose tissue of other species of mammals, including sheep and goats, dogs, cats, rabbits, swine, rats, guinea pigs and other rodents, is entirely or almost entirely devoid of pigment. In the cases of pigmented adipose tissue of cattle Palmer and Eckles (1914b) found the

pigment to be chiefly carotin, with some admixed xanthophylls. In the case of the adipose tissue of the horse van den Bergh, Muller and Broekmeyer (1920) found carotin exclusively. The latter investigators have made the only examination of human adipose tissue. Varying amounts of pigment and varying proportions of carotin and xanthophyll were found in numerous specimens obtained on autopsy of individuals dead of various disorders. In most cases carotin was somewhat in excess of xanthophyll. Of interest in this connection is the observation of Krukenberg and Wagner (1885) of a yellow lipochrome in human bone marrow. The position of the spectroscopic absorption bands of the pigment which are shown in a drawing by these authors resembles xanthophyll rather than carotin inasmuch as the maximum absorption of the first band is on the violet side of the F line, while the maximum absorption of carotin, as we now know, is at the F line.

Internal organs. As van den Bergh, Muller and Broekmeyer (1920) have shown in their extensive study of carotinoids in the human and animal body, certain of the internal organs of mammals appear to have an elective affinity for carotinoids which is greater than can be explained by their fat content. Krukenberg (1885b) first called attention to the presence of lipochrome in human and animal adrenals, at times in high concentration in the human glands. He described its extraction with hot alcohol, its absorption bands resembling those of the lipochrome of cattle serum, and the color reactions with con. H_2SO_4 and HNO_3. This was confirmed for the human adrenals by Lubarsch (1902), Sehrt (1904) and Hueck (1912). Sehrt concluded that the lipochrome was different from the plant lipochromes (carotinoids). Findlay (1920) and also van den Bergh, Muller and Broekmeyer (1920) have examined the pigment of human adrenals from the standpoint of carotinoid properties. Both carotin and xanthophylls were demonstrated, the latter authors reporting data for a large number of cases. The technic of Findlay is to be criticized, however, in that he drew his conclusion regarding both types of carotinoids being present by applying the phase test for the carotinoids directly to the issues. In other words, he regarded pigments which did not readily dissolve out of the tissue with petroleum ether as xanthophyll, while that which was extracted by this solvent he regarded as carotin. It is doubtful whether the phase test can be applied except to solutions of the carotinoids.

Van den Bergh, Muller and Broekmeyer examined the suprarenals of the horse, guinea pig, cat, dog, and swine for carotinoids. They

report carotin in all cases, the amount varying from relatively large amounts in the case of the horse and guinea pig to very little in the case of swine, cats and dogs. Findlay reported a small amount of carotin-like pigment in the suprarenals of the sheep. Palmer and Kennedy (1921) were unable to find any pigment soluble in alcohol or petroleum ether in the suprarenals of albino rats.

There has been very little study of the pigments of mammalian liver from the standpoint of carotinoids. It is difficult tissue to examine because it is rich in pigments of unknown character which are soluble in certain of the fat solvents, and also because one of these pigments, at least, gives a color reaction with con. H_2SO_4 which may easily be mistaken for a carotinoid reaction. It is to be expected that the liver of animals whose blood and adipose tissue may be rich in carotinoids will also contain these pigments, e.g., that the human liver will contain varying amounts of both carotin and xanthophylls, and that the liver of the cow and horse will contain carotin. Van den Bergh, Muller and Broekmeyer (1920) have found this to be the case. On the other hand the statement of these investigators that the liver of swine, cats, dogs and guinea pigs contains small amounts of carotinoids is to be accepted with reserve until the experimental evidence for this statement is extended to include the spectroscopic and adsorption properties of the pigments isolated. These investigators based their conclusions on color reactions with concentrated acids and upon solubility in carotinoid solvents and upon the phase test. None of these properties is properly to be regarded as specific for carotinoids.

Nerves. Meschede (1865, 1872) first observed yellow pigment in nerve cells which could be extracted with fat solvents. Rosin (1896) first associated the pigment with the lipochromes then rising into prominence. He noted its presence in the human and in cattle, and its absence from the nerve cells of the dog, cat, rabbit, rat and mouse. Rosin and Fenyvessey (1900) noted that the pigment was absent from the nerve cells of the new born, but that it was always present in the nerve cell tissue of adult humans. Following the studies of Lubarsch (1902), who regarded the lipochrome as an "abnutzung" (wear-and-tear) product of endogenous origin, pathologists have attached significance to the increase in lipochrome pigmentation in nerve cells which have been observed in disease. Dolley and Guthrie (1919), however, have made a careful study of the occurrence of chromolipoid in the nerve cell of man and animals and have found that it can be

demonstrated microchemically only in those species of animals in which carotinoids normally occur, e.g., man, cow and fowl. The conclusion which they drew seems incontrovertible, namely, that the chromolipoids of these tissues are true carotinoids of exogenous origin, the type of pigment being governed by the species of animal, i.e., by the type of carotinoid resorbed.

Lipochrome pigment is also present in other body tissue of man, i.e., in the seminal vesicle (Maass, 1889) and in the epithelial muscle cells (Akutsu, 1902) and in the heart, where Dolley and Guthrie (1921) have shown its carotinoid nature. Of interest is Akutsu's observation that the pigment is absent from these tissues in the case of new born babies and young children, and begins to occur about the age of puberty.

Skin. The skin of dairy cattle, especially that of the Jersey and Guernsey breeds, is often characterized by a high yellow color, which is often almost orange in hue. The wax in the ears of these animals is also highly pigmented. The skin color is especially noticeable on the udder, particularly the escutcheon. Using the ear wax as the source of material, Palmer and Eckles (1914b) found the pigment to be carotin, chiefly, with a little xanthophyll.

Smith (1893) observed a yellow pigment in the "dandruff" of the horse, which he regarded as modified chlorophyll. Inasmuch as he observed a variation in the amount of this pigment with the food of the animal the conclusion seems obvious that the pigment was carotin, which characterizes the blood serum and adipose tissue of this species.

Carotinoids may also color the human skin. Moro (1908), Kaup (1919), Stöllzner (1919), Klose (1919) and Hess and Myers (1919) noted skin coloration in children after eating heavily of carrots. Most of these writers associated the coloration with the carotin in the carrots, but all of them, except Hess and Myers, regarded the phenomenon as an abnormality. The latter, only, pictured the phenomenon as an exaggeration of a normal condition and demonstrated the carotin in the blood serum. They state that the feeding of oranges or eggs to children may result in a similar skin coloration. Schüssler (1919) and Salomon (1919) have noted similar phenomena in adults [2] the entire body being affected in the cases cited by Schüssler. These were

[2] Hashimato (1922) states that a yellow skin pigmentation of dietary origin was described in the Japanese literature by Baelz as early as 1896 and called, "aurantiasis cutis." It is also pointed out that Miura (1917), a Japanese writer, ascribed the pigmentation to carotin and used the term, "carotinosis." Hashimato reports 35 cases among adults as the result of excessive eating of squash.

due to a carrot diet. Carotin was inferred, not demonstrated, in these cases, although Salomon measured the extent of the "xanthemia" in certain individuals by determining the extinction coefficient of the absorption bands of the ether extract of the blood.

Von Noorden (1904) first called attention to a frequent yellow skin coloration in diabetics, which was not due to jaundice. He proposed for it the name Xanthosis Diabetica. Van den Bergh and Snapper (1913) also called attention to the phenomenon and showed in addition that it was accompanied by an increased lipochrome content of the blood serum, which they regarded as the cause. Umber (1916) noticed the same correlation in cases of Xanthosis Diabetica. Bürger and Reinhart (1918) first suggested an exogenous origin of the pathological phenomenon, for which they later (1910), as well as Salomon (1919), offered proof. Hess and Myers (1919) saw the correlation between the pathological and normal skin colorations on carotinoid rich diets, and van den Bergh and Muller (1920) and van den Bergh, Muller and Broekmeyer (1920) have presented such extensive data on the presence of carotinoids in the human organism that their conclusion seems entirely justified that the skin colorations of diabetics is due primarily to the vegetarian character of the diet of persons afflicted with this disease. It is not to be inferred, however, that carotin is always the cause of the skin coloration. Head and Johnson (1921) with the assistance of the writer have demonstrated carotin as the sole cause of one case where the diet of the diabetic was rich in carotin (the patient ate heavily of carrots), the skin clearing up when the source was removed. On the other hand another case of skin coloration of a diabetic has come under the observation of the writer which was evidently due largely, is not entirely, to xanthophylls. The diet was rich in xanthophylls (eggs and green beans), and the blood serum showed much xanthophyll with little carotin. The skin in this case was also cleared up by removing the source of the pigment.

In view of the fact that carotinoids have been found in the skin of both normal and diseased persons, it seems doubtful whether any pathological significance can be attached to its appearance in the skin. Van den Bergh, Muller and Broekmeyer (1920), who have studied this question extensively, were unable to note any correlations between the pigmentation of various tissues and the character of the disease. The difficulty, of course, is that the pigments must be of dietary origin. Even a diabetic could not show a xanthosis unless his diet contained carotinoids. On the other hand the more frequent observation of an

epidermal xanthosis in diabetics than in well persons, and the fact that a yellow color, presumably of the same origin, is frequently seen in the palms of the hands and on the soles of the feet of persons with acute sickness,[3] may have a secondary origin. The normal cause of the disappearance of carotinoids from both plants and animals is an oxidation. This is undoubtedly their ultimate fate in animals unless they are secreted in the milk fat or egg yolk (in fowls) or stored up as adipose tissue and thus protected from oxidation. Where the oxidative tone of the body is low, as in diabetes, coupled in many cases with abnormally large intake of carotinoids, it is not surprising that the pigments should appear in the tissues in abnormally large amounts. This is especially likely to be true of the epidermal tissues inasmuch as the effect of eating carotinoid-rich diets in normal persons shows that the subcutaneous glands can serve as an excretory medium for these pigments.

Carotinoids in Birds

The chromolipoid pigments of birds offer many of the most interesting problems in the field of animal chromatology. This is true in spite of the fact that a cursory knowledge of the present status of the question of carotinoid pigmentation in the case of the domestic fowl would lead one to believe that the character of the carotinoid pigments found in the feathered animals, as well as the origin of the pigments, has been settled for all species of birds. This belief is not justified. Who knows, for example, whether or not numerous species lack carotinoids entirely, as is the case, or nearly so, with many domestic mammals? This is a relatively simple problem to solve. But what shall one say of the problem of determining why the type of carotinoid in the hen is different from that of the cow; or of the problem of ascertaining why the xanthophyll of the yolk of the hen's egg appears to be chemically an isomer of plant xanthophyll in spite of the fact that the plant xanthophyll is the source from which the hen derives the pigment for the egg yolk; or of the problem of explaining the wide variation in the appearance of carotinoid in the epidermis of fowls, in all of which the adipose tissue is highly colored with xanthophyll, as well as the egg yolk? What might be expected to be simply physiological problems in connection with the behavior of carotinoids in the animal organism turn out to be complicated, or at

[3] Van den Bergh, Muller and Broekmeyer (1920) state that this phenomenon has been described for a long time by French physicians under the name "signe palmaire."

least baffling. For example, it would not seem unlikely that xanthophyll, readily soluble in fat, would act like a fat dye in the body of the hen. However, when Palmer and Kempster (1919b, c) fed Sudan III to a carotinoid-free cockerel, the dye quickly appeared in the adipose tissue and bone marrow, but not in the visible skin parts (shanks, beak, ear lobes, etc.), whereas xanthophyll, when fed to a carotinoid-free cockerel of the same breed appeared in the shank skin within 72 hours, and annatto, a different fat dye, did not appear in the body at all. Again, when Sudan III was fed to a laying carotinoid-free hen, the dye quickly appeared in the egg yolks and deeply stained the adipose tissue, whereas xanthophyll, when fed to a carotinoid-free laying hen appeared only in the egg yolk, the adipose tissue and epidermis being unaffected *even after a month of xanthophyll feeding*. How are these interesting observations to be explained?

Egg yolk. It is to be expected that the pigment of the yolk of hen's eggs should be the first of the bird chromolipoids to attract the attention of the physiologists. Städeler (1867) was the first to attempt to secure crystals of the pigment. He failed to do so, but observed the solubility of the pigment in ether and chloroform with a golden yellow color, in CS_2 with an orange color, its unsaponifiability, and the fact that HNO_3, containing NO_2, imparted a dirty blue-green color to the impure pigment, while a trace of con. H_2SO_4 had the same effect. Thudichum (1869), as already mentioned, included the pigment among his luteins. Capranica (1877) mentioned having noticed the similarity in properties of the pigment with that of the corpus luteum. The first detailed description of the spectroscopic absorption properties of the egg yolk pigment was given by Kühne (1878). Careful drawings of the pigment spectrum in ether, petroleum ether and CS_2 in comparison with a similar spectrum of the corpus luteum pigment, show differences now readily explained in the light of our knowledge regarding the type of carotinoid involved in each case. Kühne, as already mentioned, decided against an identify of the two pigments on spectroscopic grounds and also because the egg yolk pigment failed to give the blue color reaction with iodine previously noted for the corpus luteum pigment. Of interest is Kühne's observation that the egg yolk pigment is soluble in bile. Palmer and Eckles (1914d) have attached some significance to the fact that plant xanthophyll is soluble in bile (ox) while carotin is not, as a possible explanation of some of the physiological differences between these types of carotinoids in the animal body.

The probability of a definite chemical relation between the egg yolk pigment and plant carotinoids was pointed out for the first time by Schunck (1903) who found that the spectrum of the alcoholic solution was identical with one of the xanthophyll group of pigments which he isolated from a number of flowers. Schunck's work is described in Chapter II. It was pointed out there that Schunck's method for separating the xanthophylls is not exact. In all probability, however, the L xanthophyll which he described, and which showed the same spectroscopic properties as the egg yolk lipochrome, corresponds best with Tswett's α xanthophyll. This appears to be the xanthophyll which is present in the chloroplastids in greatest amount.

The definite chemical identification of the egg yolk pigment of hen's eggs as xanthophyll soon followed, when Willstätter and Escher (1912) isolated the crystalline pigment and showed that it corresponds in all its chemical and physical properties, except its melting point, with the crystalline xanthophyll of green plants. The failure of the egg yolk xanthophyll to correspond in its melting point with the plant xanthophyll of Willstätter and Mieg (1907) has never been explained. Serono (1912) has criticized Willstätter and Escher's work severely, and expressed the opinion that the product which they isolated was not a carotinoid at all, but a cholesterol ester of oleic acid. He shows how the elementary composition of such an ester corresponds even more closely with the analyses of the egg yolk xanthophyll than the latter does with plant xanthophyll, and advances the belief that this explains the high melting point found by Willstätter and Escher for the egg yolk pigment. Serono's explanation of the high melting point of the egg yolk xanthophyll falls to the ground, however, in the light of the studies of the writer (1915) which showed that the egg yolk pigment is not only chemically related to plant xanthophyll but is biologically derived from it. Whether the hen's body modifies slightly the plant pigment, thus giving it a different melting point from its precursor, or whether the hen selects one of the several plant xanthophylls differing in melting point from the mixed product obtained by Willstätter and Mieg from nettle leaves, or whether the difference is to be explained on other grounds cannot be decided definitely at the present time.

Xanthophyll is not the only carotinoid in the yolk of hen's eggs. In their isolation of the crystalline pigment Willstätter and Escher noticed the presence of a small amount of pigment with the solubility relations of carotin. The writer (1915) was able to confirm this in his

study of the biological origin of the egg yolk xanthophyll. In Willstätter and Escher's study, however, the bulk of their carotin-like pigment was saponifiable, so that its actual identity with carotin remains doubtful.

Xanthophyll pigmented egg yolks may not be normal for all species of birds. Krukenberg (1882m) examined the yolk of the eggs from two breeds of parrots. The yolk was colorless in one case, but the other was weakly tinted with a pigment whose spectrum is unquestionably that of xanthophyll.

Body tissues. It is not to be expected that animals whose eggs are highly colored with carotinoid should be devoid of the pigment in their body tissues. Halliburton (1886) showed that the blood serum and adipose tissue of the hen, pigeon and dove contains lipochrome, but the descriptions given do not make it possible to decide the character of the carotinoid involved. The writer has observed that the pigment in pigeon serum may be extracted by shaking with ether, which fact may indicate its xanthophyll nature. Schunck's (1903) spectroscopic studies included the pigment of the hen's blood serum. The same xanthophyll was found as in the egg yolk. The writer's (1915) study of the fowl's blood also showed xanthophyll to be the major pigment present in the serum, directly extractable with ether in all cases in his work.

Krukenberg's (1882b) spectroscopic drawings of the yellow skin pigments of pigeons, hens and geese resemble very closely the known spectra for xanthophyll. We now know that this pigment is xanthophyll, at least in the case of fowls, the extracts showing the phase test and spectroscopic properties of this pigment. Van den Bergh and Muller (1920) and van den Bergh, Muller and Broekmeyer (1920) have confirmed these observations with the exception of the direct extraction of the xanthophyll from fowl serum by ether. In only two out of 13 cases were they able to shake the pigment out with ether. The explanation of this divergence in their observations from those made by the writer is not at present apparent.

Retina. It has been known since the early observation of Hannover (1840) that globules varying in color from red to greenish-yellow occur in the retina of the eyes of many animals and birds. The physiologists were greatly interested in these pigmented globules during the latter half of the 19th century, particularly as to the possibility of their being related to the so-called visual pigments of the eye. These colored globules interest us, however, only in so far as the character

of the pigments is concerned. Unfortunately no modern investigation of these pigments has been made, so that it is necessary to rely on the observations of those who were unfamiliar with the possibility of their being related to plant carotinoids. The blue color reaction of the lipochromes with iodine was introduced by Schwalbe (1874) in connection with these retinal pigments. The splendid early work of Capranica (1877) on the chromolipoids of the corpus luteum and egg yolk was undertaken primarily to study the yellow to red retinal pigments of amphibians and birds. He found a complete correspondence between the retinal pigments of birds and those of the egg yolk.

Kühne contributed several papers on the retinal pigments, which appeared in the memoirs of the Physiological Institute of the University of Heidelberg. Reference has already been made to the only one of these papers which has been accessible to the writer (Kühne, 1878), which is presumably the only paper reporting Kühne's study of the chemical and physical properties of the pigments. According to this investigator the microscope reveals oil globules of three colors in the retinal epithelium of fowls, namely red, yellow and greenish-yellow. Kühne's study of these globules led him to conclude that three distinct pigments were involved, which he called rhodophane, xanthophane and chlorophane, respectively. The evidence for the existence of three pigments was based on the observations: (1) that when a dry sodium soap was prepared of the orange-red ether extract of the retinas and submitted to successive extractions with petroleum ether, ether and benzene until each solvent extracted no more pigment, the extracts were, in succession, yellowish-green, orange and rose-red in color; (2) the chlorophane in the yellowish-green petroleum ether could be purified from admixed xanthophane by repeated evaporations and extractions with petroleum ether, giving solutions more and more green in color; (3) the xanthophane in the orange ether extract could be purified from admixed chlorophane by treating the ether residue with petroleum ether (not, however, without some loss of xanthophane), and from admixed rhodophane by treating the chlorophane-free xanthophane with CS_2, in which the rhodophane was not soluble; (4) spectroscopic examination of the purified pigments showed marked differences, the chlorophane showing two bands, the other two pigments only one. It is difficult to decide from these and other less important points mentioned, what kinds of carotinoids are involved. Save for the green color emphasized by Kühne, one might be led to believe in the light of our present knowledge that his chlorophane is

the carotin which is present in traces in fowls. Some basis for this is given by the fact that its CS_2 solution was orange colored. On the other hand, the spectrum of this pigment, both in ether and CS_2, as shown by Kühne, resembles xanthophyll rather than carotin. Kühne's xanthophane would seem to be the usual xanthophyll met with in fowls, in spite of the one-banded spectrum pictured for it. The rhodophane is obviously not a carotinoid in the sense in which this term is now applied. Whether it is a decomposition product, which, in fact, Waelchi (1881) believed to be the case for all of Kühne's pigments, or another type of pigment, related perhaps to the carotinoids, cannot be decided from the meager evidence at hand. Perhaps this is the same pigment which Wurm (1871) extracted with chloroform from the wattles and "roses" (red warty spots over the eyes) of pheasants, and called tetronerythrine.

It might be mentioned in concluding the reference to Kühne's work that he was unable to observe similarly colored globules in the retinal epithelium of man, cow, pig or snakes, but he did observe that the same three pigments appear in the pigeon retina as in the fowl. Certain observations respecting the eye pigments of frogs will be referred to presently, in connection with the carotinoids in amphibia.

Feathers. Nowhere among the vertebrates does pigmentation and color attain the brilliancy and variety that is seen in the feathers of birds. Lovers of bird life, in general, as well as ornithologists, have long been interested in the phenomena. The whole range of brilliant as well as less conspicuous colors seems to be due to pigments of three colors, namely, red, yellow and black, together with the structural colors blue [4] and white. Various combinations of these pigments and colors appear to be entirely responsible for the effects observed. It is true that Gadow (1882) speaks of structural yellow in birds' feathers, but the absence of yellow pigment in these cases does not seem to be proved. Besides, the colloidal theory of optical or structural color seems to preclude yellow among such colors. Of the three common pigments involved, black is undoubtedly melanin. Since the two remaining colors due to pigment are those met with among carotinoids, interest is at once aroused as to the possibility of the carotinoids being involved. Unfortunately no modern investigation has been made of these pigments with this point in mind, with the exception of the observations of Palmer and Kempster (1919b, c) showing that the

[4] That blue color in feathers is an optical and not a pigmented color seems to have been recognized first by Bogdanow (1858).

cream color in fowls of the white feathered, normally yellow-shanked breeds is due to deposits of xanthophyll in the feathers.

At the same time it should be stated that Krukenberg (1881b, 1882a, b, m) made an extensive study of feather pigments from the point of view of his lipochromes, and inasmuch as most of his observations included spectroscopic examinations, as well as solubility and the color reactions with con. H_2SO_4 and HNO_3, it is possible to draw some inferences from his work which are of value in answering the question in hand.

Krukenberg confined his attention almost entirely to the brightly colored birds, including parrots, woodpeckers, the birds of paradise, the flamingo, cardinal, the tigerfinch and bullfinch and numerous other individual species. His studies led him to distinguish between five red pigments and five yellow pigments. Not all of these can be regarded as lipochromes, even in the sense in which Krukenberg used the term, and only a few can be considered specifically as carotinoids with the evidence given. There may be reasonable doubt, also, whether Krukenberg was justified in considering each of the pigments as separate entities. It should be stated, however, that Krukenberg, himself, was aware of this.

Of the red pigments, the most important were zoonerythrine, previously named by Bogdanow (1858) and rhodophane, previously named by Kühne (1878). Of the others, "araroth," found in the red, orange and yellow feathers of the great red macaw, *Sittace Macao,* and the yellow and orange feathers of *Aprosmictus melanurus,* is probably identical with zoonerythrine, as Krukenberg, himself, suggested. The two remaining red pigments, zoorubin and pseudozoorubin, found in the male birds, *Paradisea papuana* and *P. rubra,* are not even lipochromes in the broad sense.

Krukenberg believed that zoonerythrine was a rhodophane compound, the character of which is not stated. The properties are widely different from those described by Kühne for rhodophane, giving deep orange solutions in all the fat solvents, which readily extracted the pigment from the finely divided feathers, especially after several days digestion with alkaline trypsin or pepsin-HCl. The blue color reaction with con. H_2SO_4 was given, but the solutions showed no spectroscopic absorption bands, only a continuous absorption beginning in the green. Because of this failure to show absorption bands one is perhaps justified in concluding that the pigment is a carotinoid, altered either by the animal body (Krukenberg, himself, advanced the idea

that it was derived from the yellow pigment which colors the adipose tissue of many birds) or by the methods which Krukenberg found it necessary to use in extracting the pigment from the feathers. It is well known that the spectroscopic properties of the carotinoids are among the first to be affected adversely.

Zoonerythrine was found to be the cause of the color of the red feathers of the following birds: *Calurus auriceps, Catinga coerulea, Phoenicopterus antiquorum* (flamingo), *Cardinalis virginianus* (the cardinal bird), *Pyrocephalus rubincus, Phlegoenus cruenta* (the dagger-stab pigeon of Luzon), *Trogon Massera, Paroaria cucullata, Picus major, Pyrrhula vulgaris* (bullfinch), tigerfinch, *Megaloprepia magnifica, Cymbyrhynchus makrorhynchus,* and possibly *Ithaginus cruentatus.* The red feathers of the parrots, *Eclectus polychlorus* and *Cacatura roseicapilla,* contained the pigment as did also the yellow feathers of the bird of paradise *Xanthomelus aureus.* In addition, Krukenberg (1882m) lists 14 species of *Picides* (woodpeckers) whose red pigment is rhodophane.

Among the yellow feather pigments Krukenberg mentions zoofulvine, coriosulfurine, paradiseofulvine, picofulvine and psittacofulvine, believing, as is evident from the names, that the birds of paradise, the woodpeckers and the parrots contained, in some cases, special yellow pigments besides the general ones listed first. Of these the special parrot pigment, psittacofulvine, is evidently not even a lipochrome in the broad sense, from the description given. Paradiseofulvine, found in the yellow neck feathers of the male *Diphyllodes magnifica,* and the yellow head, neck and back feathers of the male *Paradisea papuana* and *P. rubra,* was extractable only after digestion of the feathers with alkali or trypsin. Save for complete absence of absorption bands it was identical with coriosulfurine. These facts suggest that the treatment necessary to extract the pigment altered its spectroscopic properties, a supposition confirmed by Krukenberg's own observation that heating zoofulvine in an alkaline fluid destroyed its absorption bands.

The properties of zoofulvine and coriosulfurine are so nearly identical, differing only by a slight shift in absorption bands, that their separate entity is very improbable. Krukenberg believed that the former was derived from the latter. Both pigments were readily extracted from the finely divided feathers by hot alcohol or fat solvents. Krukenberg stated that coriosulfurine withstood saponification better than zoofulvine but gave a less distinct color reaction with con. H_2SO_4.

Both were very sensitive to action of light. The position of their absorption bands, as pictured by Krukenberg, is practically identical and resembles xanthophyll very strongly. The identity of the zoofulvine and egg yolk spectra was noted by Krukenberg himself, and inasmuch as coriosulfurine is stated to be the pigment found in the beaks, shanks, skin and fatty tissue of fowls and geese, as well as in certain feathers, there is no reasonable doubt left that the two pigments are the same and are none other than the xanthophyll met with in fowls.

The birds whose feathers owe their color to this xanthophyll are as follows: The yellow feathers of *Euphone nigricollis*, the golden feathers of *Oriolus galbula*, the yellow feathers of the canary *Fringilla canaria*, the yellow feathers of the parrot *Aprosmictus melanurus*, the yellow and green feathers of *Certhiola mexicana*, and *Chlorophanes atricapilla*, the green feathers of the male parrot *Eclectus polychlorus*, the orange feathers of the great red macaw *Sittace Macao*, the yellow ornamental feathers of the male *Paradisea papuana*, the yellow and orange feathers of *Xanthomelus aureus* and *Selencides alba* and the feathers of the woodpeckers *Chrysoptilus punctigula*, *Chloronerpes aurulentus*, *C. Kirkii*, *Dendropicus cardinalis*, *Campethera nubica*, *Tiga tridactyla*, *Dryocapus auratus*, *Colaptes auratus*, and *C. olivaceus*.

The yellow picofulvine described by Krukenberg (1882m) in a number of species of woodpeckers, differs from the pigments just described in its yellowish-green color in ether and $CHCl_3$, in its orange (not red-orange) color in CS_2, in its lower solubility in petroleum ether, and by the fact that its absorption bands are in a characteristic position, shifted so greatly towards the violet from the bands of coriosulfurine that error of observation seems excluded. One is reminded strongly of the xanthophyll β of Tswett, and is tempted to suggest, provisionally, that a concentration of this carotinoid in the feathers of these birds is responsible for the pigmentation.

Carotinoids in Fishes

Observations on the pigments of fishes have been confined almost entirely to those of the skin, namely, to the causes of surface colors. The body tissues have been examined in only a few instances. The surface colorations may be likened in many respects to the feather colorations of birds. The pigments involved appear to be almost wholly reds, yellows and blacks, combined (in a physical sense) in

various ways and also with structural blues and whites. As in birds the various shades of brown are melanin combinations with reds and yellows and the greens are combinations of yellow pigment and structural blues. The structural whites, however, do not appear to be colloidal phenomena as in the case of birds, but are due to shiny crystals of guanin. Another marked difference between the surface colorations of birds and fishes is the deposition of the pigments in the latter in chromatophors over which there is nervous control such that a partial or complete contraction of the tissues makes it possible for the animal to undergo marked changes in color. This physiological phenomenon is shared by a number of other lower animals, both among the vertebrates and invertebrates. One can find the whole subject considered most exhaustively by Fuchs (1914).

As in the case of birds this monograph can deal only with the red and yellow pigments. It may be stated at the outset that a most promising field for investigation from the point of view of our present knowledge of carotinoids is offered by these pigments. No investigation whatever has been undertaken since the recent developments in this field. However, there is no reasonable doubt that the yellow pigments, at least, are carotinoids, either carotin or xanthophylls or both. What is needed especially, besides an exact determination of the carotinoid character of the yellow pigments, is a study of the red pigments whose solubilities and color reactions with the mineral acids are those of the carotinoids, but which have failed to show absorption bands in the hands of previous investigators.

De Merejowski (1881) first called attention to a rather widespread occurrence of the red pigment in fishes, under the name of tetronerythrine. He later (1883) enumerated some 20 species in which he had found the pigment, in this paper adopting Bogdanow's (1858) name zoonerythrine. No spectroscopic observations were made. The orange color in the usual fat solvents was noted, as well as the fiery red color in CS_2, the color reactions by the strong mineral acids, and the bleaching in the air and sunlight. It is interesting that de Merejowski expressed the opinion that the same pigment caused the color of carrots, tomatoes and pimentoes. Carotin, according to him, it may be noted, is a water-soluble pigment from carrots and tomatoes.

Krukenberg (1881a) first noticed the red zoonerythrine in fishes in the tailfin of *Luvarus imperialis*, the microscope showing the red granular deposits in the epithelial cells. On extraction with fat solvents or hot alcohol the pigment confirmed the observations of

de Merejowski, and in addition failed to show any absorption bands. Krukenberg (1882e) later found the same pigment in the skin of the goldfish, *Cyprinus auratus* and *Cyprinus Carpio* and (1882n) in the skin of *Mullus barbatus*. The red lipochrome in the latter fish, and that which Krukenberg and Wagner (1885) extracted from the red salmon muscle, yielded a pigment on saponification which showed the single absorption band at F of Kühne's rhodophane. It will be remembered that Krukenberg found the same pigment in the feathers of certain birds. MacMunn (see Cunningham and MacMunn, 1883) later found it in a number of other fishes. In color, the pigment resembles carotin most closely. Its relation to this pigment should be determined.

Krukenberg (1882e, n) first noted the yellow pigments in fishes, extracting them from *Cyprinus carpio*, where they were present in the skin along with the red chromolipoid, and from the skin of *Barbus fluviatilis*, *Muraena Helena*, *Belone rostrata*, *Scorpoena scrofa*, where they existed free from red pigment, and from *Mullus barbatus*, which contained the red pigment, as already noted. The absorption spectra of these pigments show their carotinoid nature, but it is difficult to decide whether carotin or xanthophyll is the predominating pigment, in view of the possibility that both types of carotinoid were present in the solutions.

The much more extensive observations of MacMunn (Cunningham and MacMunn, 1893) on the chromolipoids of the skins of a number of other species of fishes, which are accompanied by measurements of the absorption bands in ether, chloroform and carbon disulfide, are somewhat more instructive. A comparison of the data with known measurements of the absorption bands of the carotinoids leads to the following tentative conclusions: Carotin is the chief carotinoid in the skin of the Flounder (*Pleuronectes flesus*), the Plaice (*P. platessa*), the Dab (*P. limanda*), the Merry Sole (*P. microcephalus*), of *Solea variegata*, and of the Smelt (*Osmerus eperlanus*); xanthophyll is the chief carotinoid in the skin of *Arnoglossus megastoma*, *Trigla cuculus*, *Trigla hirundo*, the Mackerel (*Scomber scombrus*), *Syngnathus acus*, *Siphonostoma typhle*, *Clupea narengus*, *Artherina presbyter*, the John Dorey (*Zeus faber*) and the fifteen spined Stickleback (*Gasterosteus spinachia*); both carotin and xanthophyll are found in *Cottus bubalis*, and the banded Pipe Fish (*Nerophis oequoreus*); a pigment whose spectra strongly resembled lycopin is the cause of the skin pigment of the goldfish, *Cirassius auratus*.

One cannot leave the paper of Cunningham and MacMunn without referring to the interesting experiments of Cunningham on the colorless side of flounders. These interesting fish, as is well known, have the habit of lying continuously on their left sides as near as possible to the bottom of the sea, or the tank in which they may be placed. The lower side of the fish is almost always devoid of the black and yellow color which characterizes the upper side of the fish. Cunningham, however, was able to cause the fish to develop normal pigmentation on both sides by placing them in a tank with a glass bottom with a light reflecting mirror below it so that the fish were exposed to daylight on both sides. Cunningham and MacMunn naturally concluded that it is light which causes the deposition of pigment in the flounder's skin. There seems to be nothing to discredit this conclusion so long as one accepts as proved that pigment is actually absent from the colorless side of the flounder and that chromatophors in the epithelial tissues play no part in the phenomenon.

With regard to chromolipoids in other tissues of the fishes, information is almost completely lacking. We have the observations of Krukenberg and Wagner (1885) already referred to, of a red zoonerythrine in salmon muscle, changing to a rhodophane on saponification. We also have the statement of MacMunn (1883) that the liver of fishes may contain a tetronerythrine (zoonerythrine). Finally, we have Miss Newbigin's (1898) examination of the red pigment in salmon muscle, in which she found a yellow non-lipochrome pigment as well as the red lipochrome, showing the usual lipochrome reactions save the absorption bands. She believed that the red pigment readily formed compounds with sodium and potassium, which could be decomposed with acetic acid. The yellow pigment was soluble in the fat solvents, did not form compounds with sodium and potassium but failed to show the color reactions with concentrated acids. The spectroscopic absorption properties apparently were not observed. The same red and yellow pigments were also present in the ovaries of the mature female.

Carotinoids in Amphibians

The phenomena governing the coloration of these vertebrates, as well as the colors observed, are almost identical with those of fishes. As in the case of the fish pigments, the chromolipoids offer an interesting problem for study from the newer point of view of these pigments. The observations which have been made from the older lipo-

chrome point of view have been confined to the frog and salamander.

The alcohol and ether extractability of the yellow pigment in frogs was known to the early observers, such as v. Wittich (1854), Leydig (1868), Hering and Hoyer (1869), before Capranica (1877) found that the retinal pigment of the frog corresponded in its general solubility, chromatic and spectroscopic properties with the corpus luteum and egg yolk pigment. Kühne's (1878) chromophane studies included the pigment in the retinal and adipose tissue of frogs, as well as the skin. Only one pigment was found, readily and completely extractable from the saponified extracts with petroleum ether. On account of a slight spectroscopic difference from the pigment of egg yolk (absence of a faint third band in spectrum of frog pigment) Kühne gave the frog pigment the name lipochrin.

Krukenberg (1882c) repeated Kühne's work on the yellow or orange skin pigment of the frogs *Hyla arborea, Rana esculenta,* the toads *Bufo viridis, Bufo calamita, Bufo vulgaris,* and the orange skin pigment of the salamanders, *Triton cristatus* and *Salamdra maculosa.* The same pigment was found throughout, also in the ovaries of *B. calamita* and the fatty tissue of *Triton cristatus.* A comparison of the spectral drawings of Kühne and Krukenberg for their amphibian lipochromes shows certain differences in the positions of the absorption bands such that it is impossible to decide whether the pigment is carotin or xanthophyll, so that the determination of this important point will have to be left to future investigation. It should be stated, perhaps, that Magnan (1907a, b) has claimed to have isolated a green and a yellow pigment from several *Batracian's* skins, the yellow pigment differing from the chromolipoid obtained by previous workers in that it failed to show absorption bands, and was soluble in NaOH and KOH. One cannot help but raise some doubt as to the accuracy of this worker's observations both as to the properties of his yellow pigment and the existence of a green pigment. It is an old observation that the frog skin loses its green color on extraction of the yellow pigment, showing that the green color is partly of pigment and partly of structural origin.

Carotinoids in Reptiles

The surface colorations of reptiles are perhaps even more conspicuous than those of amphibians. The lizards and snakes have been studied most, and there has been at least one observation regarding lipochromes in turtles. The deposition of the skin pigment in nerve

controlled chromatophores and the consequent power to change color has been developed to a high point of perfection in the lizards.

Among the snakes, which frequently are marked with yellow colors, lipochromes in the broad sense and thus carotinoids, in the narrower sense, do not appear to occur. Kühne (1878) noticed the absence of retinal pigments in snakes. According to Krukenberg (1882d) the yellow pigment which can be extracted after long boiling with absolute alcohol from the skin, muscles, connective tissue and fatty tissue of the snakes, *Tropidonotus natrix, Elaphis quadrilineatis* Bonaparte, *Callopeltis quadrilineatis* Pallas and *Rhinescis scalaris,* and which is soluble is ether, $CHCl_3$ and CS_2 after extraction, differs from the lipochromes in the persistent green fluorescence of its solutions, the failure tc show absorption bands or chromatic reactions, and the failure to bleach with oxidizing agents. Similarly, according to Cunningham and MacMunn (1893) the yellow skin pigment of the alligator is not lipochrome.

Among the lizards, however, the presence of ether and alcohol soluble pigments of yellow color was apparently observed by a number of workers before Krukenberg (1882d, n) first submitted them to spectroscopic examination. Using the skins of the cameleons *Lacerta muralis, Lacerta agilis, Camaelon vulgaris* and *Bombinator igneus,* the yellow and orange pigments were extracted and found to correspond completely with other yellow and orange lipochromes. The position of the two absorption bands resembled most those previously found by Krukenberg for the feather pigment zoofulvine, but because of an uncertainty in his mind as to the identity of the pigments, Krukenberg called the lizard pigment lacertofulvine. In all probability the pigment is xanthophyll, or at least one of this group of carotinoids.

Among turtles we have the observation of Halliburton (1886) that the blood serum and adipose tissue of the tortoise is rich in a lipochrome showing the spectroscopic and other lipochromatic characters of the blood serum and adipose tissue pigments of the hen. The question needs further study, however, before it can be even regarded as probable that this pigment is xanthophyll.

Summary

Piccolo and Lieben (1866) and Holm (1867) isolated the first animal chromolipoid in pure condition, namely, the pigment of the corpus

luteum of the cow. Although the general relation of this pigment to other yellow animal pigments, and even to certain of the plant chromolipoids was recognized by a number of subsequent investigators, its identity as carotin was not established until the work of Escher (1913). The character of the corpus luteum pigment in other mammals and in man has not been determined. Carotinoids are absent entirely in the case of the so-called yellow bodies on the ovaries of swine.

The existence of a chromolipoid in the blood serum of certain mammals was known as early as 1835. Krukenberg (1885a) first succeeded in isolating the pigment (using ox serum) and classified the pigment as a lipochrome. The relation of the pigment to the carotinoids which characterize other mammalian tissues was not established until the work of Palmer and Eckles (1914c). The chromolipoid of cattle and horse serum is carotin, but in man it may be either carotin or xanthophyll. Carotin, when present, is frequently, if not always, bound to colloidal serum albumin, but this does not appear to be the case for xanthophyll. Carotinoid is not always the sole pigment in blood serum, bilirubin also being present at times, particularly in the case of man and the horse. The blood serum of a number of mammals is almost or entirely devoid of carotinoid pigment under all conditions, e.g., swine, sheep, goats, dogs, cats, guinea pigs and rats. This is also true for the new-born animals of the species whose serum is pigmented in later life.

The chromolipoid of milk fat is the carotinoid which characterizes the blood plasma of the animal, as shown by the author's studies. Carotinoid coloration of milk fat is not, however, universal among mammals, pigmentation being determined by the kind and amount of carotinoid carried by the blood.

The chromolipoids of the adipose tissue, internal organs, nerve cells and skin of mammals are the carotinoids which characterize the blood serum, only those animals whose blood serum is normally pigmented with carotinoids depositing the pigments in their body tissues and organs.

The more frequent observation of an epidermal carotinoid coloration among diabetics than among well persons is due largely to the vegetarian character of the diet in diabetes, from which the pigments are derived. The author suggests, however, that the phenomenon is due also, in part at least, to the lowered oxidative tone of the body in this

disease, inasmuch as oxidation is undoubtedly the normal means by which the animal body destroys surplus carotinoids.

The chromolipoid pigments of birds offer many interesting physiological problems, particularly because of their chemical difference from the chromolipoids of mammals. The egg yolk pigment was studied as early as 1867, and while Kühne (1878) first recognized that it is not identical with the pigment of the corpus luteum of mammals, its probably chemical relation to plant xanthophyll was not suggested until the work of Schunck (1903). This relation was established by Willstätter and Escher (1912), and extended to include a biological relation by Palmer (1915). Whether the hen's body modifies slightly the plant xanthophyll or selects one of the several plant xanthophylls differing in melting point from the mixed product obtained from green leaves, cannot be decided at present.

Xanthophyll also appears to be the chief, if not the sole carotinoid in the blood serum, adipose tissue, nerve cells, body organs and skin of fowls. Detailed studies have not been made for other birds. The relation of the so-called lipochromes of the retina of the eyes of birds to the carotinoids is indefinite.

Carotinoids and related pigments are unquestionably the cause of the yellow to red color of the feathers of certain birds which are enumerated in the text. Although these pigments have not been studied since the recent advances in our knowledge regarding animal carotinoids, the evidence points to the fact that xanthophyll is one of the pigments concerned in feather coloration.

Skin coloration of fishes is similar in some respects to feather coloration in birds except that the structural whites are not colloidal in fishes and fish pigments are deposited in chromatophores over which there is physiological control. The red chromolipoid of the skin of many fishes does not appear to be identical with any of the known carotinoids although it resembles carotin in many respects and appears to be related to the red so-called carotinin found in many lower plants. The yellow chromolipoids of fishes are no doubt true carotinoids, the evidence available, based on older observations of Krukenberg and MacMunn, indicating the presence of both carotin and xanthophylls. MacMunn has even described a lipochrome in the skin of the goldfish *Cirassius auratus,* which strongly resembles lycopin.

The chromolipoids of the blood, body tissues and organs of fishes have been examined only in the case of the salmon, in which the red

pigment of the flesh and ovaries appears to be the carotinoid-like carotinin described above.

Among amphibians the yellow pigments of the skin, adipose tissue and retina of frogs, toads and salamanders are unquestionably carotinoids, but the evidence at hand does not show whether carotin or xanthophyll or both are concerned.

Yellow pigments among reptiles appear to be more frequently non-carotinoid in nature. There is a probability, however, that a xanthophyll is the chromolipoid of cameleon skins. The blood serum and adipose tissue of the tortoise also contain carotinoids, the nature of which is not known.

Chapter V

Carotinoids in Invertebrates

The causes of the colorations and pigmentations encountered among the lower forms of animal life have not been without interest to the biologists. It is to be expected that the developments in the field of chromatology which took place during the 19th century should be accompanied by studies of invertebrate pigments by the zoologists and others interested in these forms of life. These studies have an especially important bearing on the subject of the distribution of carotinoid pigments among animals because, as has already been pointed out, evidence of a more definite nature has been presented for the existence of these plant pigments in animals of the invertebrate group, than for a number of the vertebrates. It should be understood, however, that carotinoids do not predominate among the pigments of the lower animals. On the contrary, one would hardly be justified in asserting that the carotinoids predominate among the pigments of yellow to red color encountered among the invertebrates. As in the case of plants, it appears that as one descends the scale of living forms, non-carotinoid pigments of yellow to red hues seem to be met more and more frequently. For example, it will be shown presently that carotinoids are undoubtedly abundantly present in the larvæ and pupæ of butterflies and moths, but the brilliant reds, golds and yellows seen in the butterflies themselves are apparently caused by pigments of entirely different characteristics. Again, among the crustacea and worms, other red and yellow pigments are often the cause of colorations. These facts, however, do not detract from the interest which is naturally aroused by the presence of the carotinoids in at least some species of almost all the main groups of invertebrate animals. One might think, perhaps, that the simpler digestive apparatus of the lower animals would insure a more abundant distribution of biologically derived pigments. Whether this is true or not will have be decided by the investigations of the future.

Carotinoids in Insects

Zoologists recognize as many as eleven different orders of the *Insectia,* and state that a million species—more or less—may exist in the world. When these figures are contrasted with the fact that not over thirty-five or forty species, belonging to four orders, have been examined, with reasonable indications of carotinoids or closely related pigments being present, it is seen that very little, indeed, has been done in this field.

The insect orders in which carotinoids appear to be present are the *Lepidoptera* (butterflies), *Rhynchota* (bugs), *Coleoptera* (beetles) and the *Orthoptera* (locusts, grasshoppers).

Lepidoptera. In the butterflies themselves the brilliant wing colors are not due to carotinoids, as already mentioned. There are undoubtedly some color effects which are purely structural, but the red, orange, and yellow pigments appear to be derivatives of uric acid, as shown by the investigations of Hopkins (1889, 1891, 1892, 1896) and Urech (1893). In the larvæ and pupæ, however, either carotinoids or modified carotinoids are frequently encountered.

Medola (1873) first showed that the green color of insects is not due merely to the green digestive mass in the food canal, but to a true absorption of pigment by the hämolymph (blood) of the animals, although in a somewhat modified form. This laid the foundation for the classic experiments of Poulton (1885) on the pigments of the larvæ and pupæ of a number of species of butterflies. Poulton distinguished between two kinds of pigments in phytophagus larvæ, namely, those derived from the food and those produced by the animals themselves. The general thesis which his work supports may perhaps best be explained by the following quotation. "All green coloration is due to chlorophyll; while nearly all yellows are due to xanthophyll. All other colors (including black and white, and some yellow, especially those with an orange tinge) are due to the second class of cause (so far as I am aware: It is, however, extremely probable that certain colors may be proved to arise from the modification of the derived pigments, and many observations make it probable that other colors may be derived from plants in the case of larvæ feeding upon petals, etc.). The derived pigments often occur dissolved in the blood, or segregated in the subcuticular tissues (probably the hypodermis cells), or even in the chitinous layer, closely associated with this cuticle itself."

Our interest naturally centers around the derived "xanthophylls" found in the hämolymph and other tissues, and Poulton's proof for its existence. It may be stated first, however, that Poulton used the word xanthopyll in a collective sense for the yellow pigments accompanying chlorophyll. The use of the word carotinoids conveys the same meaning. The proof for the derived carotinoids in the larvæ and pupæ rested largely upon a spectroscopic examination of the blood extracts in comparison with the spectrum of the pigments of green leaves under like conditions. Poulton's own conclusion was that the points of difference between the derived "xanthophyll" spectrum of caterpillars and that of green plants made it impossible to decide whether more than one derived "xanthophyll" was present. Interpreted from the point of view of our present knowledge of the carotinoids this means that, inasmuch as Poulton was dealing with extracts for which no purifications were attempted, it is impossible to decide whether carotin or xanthophyll or a mixture of carotinoids causes the colors of the carotinoid type found in caterpillars.

It is impossible to review Poulton's entire paper. There is one further point, however, which may throw some light on the character of the carotinoids taken up by these insects and which at least forms an interesting link between carotinoids as found in mammals and the same pigments in caterpillars. This point is the great stability of the "xanthophyll" in the blood of these insects, which led Poulton to believe that it "may be due to association with a protein of the blood." Poulton found that ether, chloroform and carbon disulfide would not extract the pigment from the blood, although the ether precipitated the blood proteins in the form of a green jelly and eventually, after some hours, became bright yellow with pigment. However, when alcohol was used as a protein precipitant, the pigments dissolved at once in the supernatant alcohol, especially if absolute alcohol was employed. If the affinity of carotin for blood protein, as found in some cases for mammals, is a universal property of this pigment, these results of Poulton's on the haemolymph of caterpillars lend support to the tentative conclusion that the chief carotinoid of the larvae and pupae of butterflies is carotin.

In view of the small number of insect species studied from the point of view of carotinoids it may be well to mention that the species of *Lepidoptera* examined by Poulton were as follows: *Pygaera Bucephalus, P. Meticulosa, Smirinthus Tiliæ, S. Populi, S. Oscellatus,*

Sphinx Ligustru, C. Elpenor, D. Vinula, Papilio Machaon, Ephyra Punctaria, and *E. Angularia.*

Krukenberg (1886) made spectroscopic observations of the pigments in the haemolymph of the pupa of several additional species of *Lepidoptera,* namely, *Platisama Cocropia, Telea Polyphemus, Saturnia Pernyi* and *Saturnia Pyri.* In these cases the haemolymph itself, as well as the alcoholic extracts, showed the lipochrome absorption bands.

In the case of *Saturnia Pyri* lipochrome was also extracted from the body tissues. The hämolymph of another species, *Collosamia Promethia,* did not show the presence of lipochrome until first extracted with alcohol. Krukenberg's observations, unfortunately, do not throw any light on the character of the carotinoids present in caterpillars, although they support strongly the idea of a general distribution of carotinoids in the blood and tissues of these herbivorous insects. This cannot be said, however, of the recent extensive study of this question by Geyer (1913). According to Geyer's own conclusions his results are in entire agreement with Poulton's as to the presence of xanthophyll in the haemolymph of the larvae and pupae of the *Lepidoptera.* In spite of an acknowledged familiarity with the work of Willstätter, Geyer compared the spectrum of ether solutions of the haemolymph pigments with extracts of yellow flowers obtained with 70 per cent alcohol. Inasmuch as this solvent does not extract the true carotinoids from plant tissues, it is not surprising that Geyer's spectrum revealed no absorption bands in the blue and green. His blood extracts also failed to show absorption bands, in opposition to the work of Poulton, so that we are still in the dark as to the exact nature of the carotinoid pigments taken up by the caterpillars. This is unfortunate because Geyer's work is sufficiently recent to have permitted him to use the technic which would have given the desired information.

While Geyer's studies are disappointing with respect to the kind of carotinoids present in caterpillars, he noticed an interesting sexual difference among certain species in connection with the pigmentation of the hämolymph. In *Bombyx mori,* the larva and pupa blood of the males was always colorless or very faintly tinted, while that of the females was always a bright golden yellow. Similarly in the females of *Xanthia flavago* the blood was yellowish green, while that of the males was colorless or very pale yellow. In other species the blood of the females was green, containing both chlorophyll and carotinoids, but the males again showed colorless or nearly colorless blood. Geyer

believes that this sexual difference is related to the ability of the females to impart the blood pigmentation to the eggs, for protective purposes, an idea previously proposed by Poulton, who also noticed the pigmentation of the eggs. Although the writer is not in sympathy with the protective notions regarding animal colorations, believing that such phenomena are to be explained entirely on physiological grounds, and not through theories built upon the assumption that colors impart the same sensations upon the retina of the eyes of lower animals that they do upon our own, it is nevertheless an interesting fact that insects apparently have the ability to impart the derived pigments found in the blood to their eggs just as is found in the case of the higher oviparous animals.

Rynchota. Among this group of insects, usually called bugs, red, yellow and green colored species are commonly encountered. Carotinoids are to be expected because the insects are mostly phytophagous. Among the *Aphids,* or plant lice, the green colors are undoubtedly derived from the food as in the case of caterpillar larvæ, as Macchiati (1883) first pointed out. Sorby's (1871c) study of this green pigment showed, however, that the yellow pigments accompanying chlorophyll are also present, and may be extracted from the crushed insects with carbon disulfide. The two well-marked absorption bands in the blue shown by these extracts, at once classifies the pigment among the carotinoids. Sorby, himself, called the pigment aphidoluteine. The pigment of some red aphids may not be carotinoid, because Sorby found that the red color of aphids which he found on apple trees could be extracted with hot water, the extract turning yellow on addition of acetic acid, and red again when ammonium hydroxide was added. The properties of the pigment suggest an anthocyanin-like substance.

Red coloration in the tegument among some species of bugs, is unquestionably carotinoid at times, perhaps carotin itself. Thus, Physalix (1894) extracted the red pigment of the hemipter, *Pyrrhocoris apterus,* from two liters of the insects. The pigment was deep red in carbon disulfide, yellow in alcohol and ether, gave the lipochrome reaction with con. H_2SO_4, and showed the absorption spectra of carotin. Physalix asserted that the pigment was carotin or a very closely related substance.

Coleoptera. Green and yellow and red pigments also characterize the tegument of the beetles. Leydig (1876) first noticed the autumnlike changes in the color of the green beetles *Cassidæ* and the species,

Carabus auratus, a phenomenon also noticed by Sorby (1871c) in the case of green *Aphids*. There can be little doubt that the yellow pigments found in these insects are often, if not always, true carotinoids. Thus, Zopf (1892b) describes the properties of the orange pigment in the wing coverings of the willow-leaf beetle *Clythra quadripunctata*, as showing all the usual lipochrome reactions (including his lypocyan reaction described in Chapter III), and the absorption bands in petroleum ether lying at 496-480μμ and 460-448μμ. Zopf called the pigment a di-carotin or "eucarotin." The absorption bands indicate carotin itself. The same pigment was found in the yolk of this insect's eggs. Schulze (1913, 1914), although he has examined the pigments less critically from a chemical point of view, has adopted the idea that the yellow and orange pigments in the wing coverings of many beetles are true carotinoids. He has examined especially the species *Melasoma XX-punctatum*, *Melasoma populi*, *Chrysomela polita* and *Chrysomela varians*. He states (1914) that the pigments appear to approach the xanthophylls, rather than carotin, in their properties, but does not present the chemical evidence for this statement.

The red pigments of the beetles appear to belong to the carotinoid-like class of pigments which have been mentioned repeatedly in the foregoing pages under the names carotinin, rhodophane, zoonerythrine, etc., which are characterized especially by showing only one absorption band at the F line. Zopf (1892b) described the properties of this pigment in the poplar leaf beetles *Lina populi* and *Lina tremulæ*, as well as the beetles *Coccinella septempunctata* and *C. quinquepunctata*. Zopf found the pigment in the wing coverings, on the abdomen, on the lateral edges and end of the back, and also in the eggs of the poplar leaf beetles. He also noticed that the latter insects secreted the pigment from the mouth when excited by handling or stimulated by chloroform. The *Coccinellæ* also secreted the pigment, but the secreting cells were found to be in the joints, not in the mouth. Zopf described the solubility of the pigment in the fat solvents and in oils, the lipochrome color reactions, including the lipocyan reaction, and the single spectroscopic absorption band shown by the ether solution at 515-480μμ. At the time of his investigation of the pigment Zopf called it a mono-carotin, but later (1893a) referred to it as Lina-carotin. Griffiths (1897) attempted to ascertain the composition of the pigment, which he called coleopterin, using the species *Pyrochra coccinea*, *Lina populi* and *Coccinella septempunctata* as the source of his material. The extracts secured, using boiling alcohol

or ether, were purified merely by repeated re-solutions and evaporations. The amorphous residue finally obtained contained 7.7 per cent nitrogen, and showed a gross composition conforming to the formula $C_7H_5NO_5$. This is the only analysis that has been made of the substance. The method employed for its purification could not be expected to prevent oxidation of the pigment (Zopf showed that the pigment readily bleached in the air) and would not insure freedom of the product from alcohol and ether soluble impurities. Griffiths' results are therefore open to question and cannot be accepted as showing the constitution of this important carotin-like pigment.

Kremer (1919) has recently objected vigorously to the use of the terms carotin and xanthophyll in connection with the lipochromes of the *Coleoptera,* and, in fact, for the lipochromes of insects in general, on the grounds that the older terminology of Krukenberg suffices until the character of the animal lipochromes has been more accurately determined. It is agreed, of course, that all scientific effort must advance along conservative lines. At the same time one cannot afford to be conservative to the point of being reactionary.

Orthoptera. The facts concerning carotinoids among insect pigments presented in the preceding paragraphs in themselves lend strong support to the supposition that similar pigments exist in the yellow, green and multicolored grasshoppers and other species belonging to this group. The few experimental observations which have been made are, however, inadequate for the proof of this supposition. Krukenberg (1880) recorded a brief study of these pigments in connection with his attempt to explain Leydig's (1876) observation that the common green locust, *Locusta viridissima,* changes to a brownish-yellow color simultaneously with similar changes in foliage in the autumn. Although he found that the chitinous layers in this species, as well as *Mirbius viridis* and the common green grasshopper contained specific green, yellow and red pigments, whose varying sensitiveness to destruction by light was the probable cause of the color changes noted by Leydig, the chemical properties of the pigments, particularly their failure to show absorption bands, necessarily leaves the question open as to the probability of carotinoid pigments being involved in their coloration. It is true that Podiapolsky (1907) found that a golden yellow solution was obtained by treating alcoholic extracts of green locusts with $Ba(OH)_2$, and concluded that the pigment thus secured was apparently identical with plant xanthophyll. Obvi-

ously, the whole matter of the grasshopper and locust pigments needs further study.

Acerata. The animals in this group are not, strictly speaking, insects, but are a lower order midway between insects and crustacea. Heim (1892) examined the red pigment in the larvae of one species, namely *Trombidium,* or common red mite. He found it to be soluble in the fat solvents with a red color and that it gave the lipochrome reaction with the concentrated acids. Its possible relation to the carotinoids is thus indicated.

Carotinoids in Crustacea

Pigmentation among the Crustacea is characterized both by the variety of colors exhibited and by their brilliancy. The various colors found, including blue, green, and various shades of orange, red and brown, are more frequently found singly on a species, rather than mixed to give varied-colored effects. Examples of brilliant single colors are seen in the higher and lower crabs, the lobster, and the crayfish. Instances of varied-colored forms are the prawns, such as *Hippolyte varians, Leander serrator,* and the wrasse, *Grenilabrus melops.* These latter species have various pigments deposited in chromatophores, whose expansion and contraction under the influence of various agents, brings about some remarkable color changes in the animals. Contrary to the situation found in many of the higher animals the blue and green colors encountered in Crustacea are not structural, but are due to pigments whose relation to the red lipochrome so common to these animals is so intimate and yet so fugitive, that its exact nature has never been discovered.

From an historical point of view Pouchet (1876) seems to have first described the properties of red and yellow ether soluble pigments in the hypodermis, eggs and ovaries of the lobster and other Crustacea. Both pigments dissolved in concentrated H_2SO_4 with a color change from green to blue to violet. The pigments differed, however, in that the yellow one was soluble in alcohol, but the red one was not. The red pigment was obtained in crystalline form, the crystals being violet colored with a metallic reflection. Jolyet and Regnard (1877) noted the presence of a yellow, ether soluble pigment in crab's blood, and Frédérique (1885) a similar red pigment in the blood plasma of the lobster. Moseley (1877) was the first to name the red pigment, calling it crustaceorubin. He also noticed its single absorption band in

the blue-green part of the spectrum. Merejowsky (1881, 1883) described the same pigment under the name zoonerythrine, and enumerated various species of Crustacea in which it occurred. Maly (1881), working with the red eggs of the spider crab, *Maia Squinado,* differentiated between a red vitellorubin and a yellow vitellolutein, showing many solubility and chromatic properties in common, but differing in their spectroscopic properties and in their affinity for alkalis. Krukenberg (1882k) included both pigments under his lipochromes, but was convinced of the identity of the red pigment with Kühne's rhodophane. Halliburton (1885) made a special study of the red "tetronerythrine" in the blood of the lobster, crab and crayfish, but noticed a difference between the fresh water (*Astacus fluviatilis*) and salt water (*Nephrops norwegicus*) form of the latter, in that the pigment was almost absent from the salt water animals. Yellow chromolipoid is not mentioned. MacMunn (1883) examined the liver and bile of lobsters, crabs and crayfish for lipochromes, finding yellow "lutein" in some cases and red "tetronerythrine" in others. Many of the investigators mentioned also made a cursory examination of the pigments in the hypoderm, but these have been studied especially by MacMunn (1890) and Newbigin (1897) for the larger species of Crustacea and by Blanchard (1890) and Zopf (1893a) for the smaller. The latter investigators used chiefly the little red *Diaptomus bacillifer,* found in fresh water, for the source of their material. Blanchard found only one pigment, but Zopf describes both red and yellow pigments, the red one being called diaptomin.

This brief historical survey makes it clear that two distinct types of chromolipoids are present in Crustacea, one characterized by its red color and the other by a more yellow hue. How are these pigments related to the carotinoids?

With regard to the red pigment its properties have been described most fully by Maly (1881), Zopf (1893a) and Newbigin (1897) as follows: The ether and petroleum ether solutions are yellow, when dilute, but the alcohol, benzene, chloroform and carbon disulfide solutions are always red or pink, even on great dilution. Water, also, acting on material such as the dried *Maia* eggs forms a protein solution in which the coloring matter is apparently dissolved, and from which the pigment can be removed by coagulating the protein with heat or alcohol and extracting the dried precipitate. The pigment is very unstable when pure and fades very rapidly in contact with air, even in darkness. This bleaching is undoubtedly an oxidation, and

at once shows the close relation of the pigment to the carotinoids. The chromatic colorations with the strong mineral acids are also identical with the carotinoid colorations under like conditions. However, unlike any of the known carotinoids it appears to form compounds with the caustic alkalis, and alkali earth metals, and can, moreover, be precipitated from its alcoholic solution on addition of saturated $Ba(OH)_2$, $Ca(OH)_2$ and $Mg(OH)_2$ solutions. The pigment can be readily liberated from its alkali and alkali earth compounds by acids, apparently without injury to its properties. According to Maly and Newbigin these pigment compounds are insoluble in alcohol, but are soluble in ether, chloroform, carbon disulfide, benzene and petroleum ether (slightly). Zopf, however, denied the solubility of the calcium and barium compounds in any of these solvents, and states that the sodium compound, only, is soluble in the solvents mentioned. He noted, also, that the sodium compound, like the free pigment, readily bleaches. Spectroscopically, as already mentioned, the pigment differs from the known carotinoids in that it shows only one absorption band. According to Zopf this band in ether lies at 515-465$\mu\mu$ and in carbon disulfide at 533-482$\mu\mu$.

In view of the unanimity of the above mentioned investigators on the properties described one cannot help being somewhat surprised at the recent announcement of Verne (1920a, b) that the red pigment of Crustacea is none other than carotin. It is stated that the pigment which he isolated in pure crystalline form from the hypodermis of the Decapod Crustacea (lobsters, crabs, etc.) has the same melting point, forms the same iodide, exhibits the same absorption spectra and shows the same composition on analysis as carotin. It is true that Blanchard (1890) called the impure red pigment which he isolated carotin, but he was hardly justified in so doing inasmuch as his pigment showed no absorption bands whatever. The findings of Verne, therefore, while very significant, cannot be given unqualified acceptance until it is possible to explain the peculiar properties which were obtained by all previous investigators for this carotin-like pigment.

As for the yellow pigment we have the observations of Maly, Krukenberg, MacMunn and Zopf, to determine its possible relation to the carotinoids. Miss Newbigin's (1897) failure to confirm the lipochrome characteristics of this pigment can only be explained on the grounds that she was dealing with a decomposition product. There is certainly no basis for her idea that the two-banded spectrum ob-

served by the other investigators was due to the action of light on the red pigment. The properties of the yellow pigment which show its probable carotinoid nature are its solubility in alcohol, ether, petroleum ether, chloroform and carbon disulfide, its color being orange red in the last named solvent; its resistance to saponification; its lipochrome color reactions with the concentrated mineral acids; its two-banded absorption spectrum; and the great ease with which the pigment bleaches. As to whether the pigment is carotin or xanthophyll we have only the measurements of the absorption bands of the "lutein" which MacMunn (1883) extracted from the liver of the crab, *Cancer pagurus,* and of the "yellow carotin" which Zopf secured from the little Diaptomus crustacean. MacMunn gives the measurements in ether as 498-480μμ and 466-450μμ, and in CS_2 as 530-507μμ and 496-476μμ. Zopf's "yellow carotin" in petroleum ether showed bands at 498-479μμ and 464-450μμ. The agreement exhibited in like solvents indicates that these investigators were dealing with the same pigment. The position of the bands suggests carotin rather than xanthophyll.

As is well known, the red color so frequently associated with Crustacea is apparently absent from the external tissues until the application of heat produces the usual brilliant red hue. The common lobster is a conspicuous example. The shell of this animal is very dark blue, although the underlying hypodermis is red. In the case of the fresh water crayfish, *Astacus nobilis,* the shell is grayish brown and the hypodermis blue. The salt water crayfish or Norway lobster, *Nephrops norwegicus*, has an orange shell and red hypodermis. Green colors are also seen, as in the species *Virbius viridis*. Blue colors are found among the Copepods, also, Merejowsky (1883) mentioning the species *Anemalocera Patersoni* and *Pontellina gigantea.* The very fugitive character of these blue colors has been known for many years. Not only heat, but reagents like alcohol, ether, or acids change the color of the tissues to the characteristic red. Pouchet (1876) believed that the phenomenon was due to the destruction of a very unstable blue pigment which then allowed previously invisible red pigment to be seen. Krukenberg (1882k), however, advanced the theory that the blue and green colors were due to lipochromogens which were transformed by the various reagents into lipochromes. This theory has been adopted by nearly all subsequent investigators, including Merejowsky (1883), MacMunn (1890) and Newbigin (1897). Merejowsky called the lipochromogen velelline, after Negri,

and describes the transformation of the blue aqueous solution into "zoonerythrine." He says, in substance, that if a filtered blue aqueous extract is treated with a drop of acid (H_2SO_4, HNO_3, HCl, acetic or picric), and then with a drop of strong KOH, NaOH or NH_4OH, and then with several drops of absolute alcohol, there is an instantaneous color change of blue to red orange. On filtering, the filtrate is colorless and the red-orange substance left on the filter gives all the properties of "zoonerythrine." Merejowsky describes a similar change for a green water-soluble "astroviridine" which he extracted from the Crustacea, *Gebbia littoralis* and *Paloemon viridis.*

Miss Newbigin (1897) likewise obtained an aqueous solution of blue pigment from the hypodermis of the lobster and the epidermis of the fresh water crayfish by suspending scrapings from the shell of hypodermis in 0.1 per cent HCl. She states, "This solution is first pink but later turns blue on standing as the solution becomes neutral or alkaline with the formation of $CaCl_2$ from the line of the shell. The blue solution is very unstable. Heat (45°—50° C.), acids, alcohol or ether turn it pink instantly. The pink pigment is readily soluble in alcohol or ether, and gives all the characters of crustaceorubin." An observation that ammonia is always given off at the conversion of blue into red suggested to Miss Newbigin that the compound of lipochrome giving the blue color is an organic base. She points out that it cannot be a simple ammonia compound because the alkali compounds of the red pigment are red, not blue.

It is clear that the true explanation of the character of these interesting chromogens has not yet been discovered. One cannot help but wonder whether there may be an analogy between these phenomena and blue colloidal gold. The sensitiveness of the blue solution to reagents which are known to aggregate colloidal particles and the precipitation of chromolipoid which occurs following the use of these reagents is certainly strongly suggestive of a colloidal phenomenon. The stabilizer of the suspensoid may well be a basic ammonia-containing substance extracted from the tissues with water.

Carotinoids in Echinoderms

The most familiar of the animals included under this group are the *Asteroids,* or star-fishes; the *Ophiuroids,* or brittle stars; the *Echinoids,* or sea urchins; the *Crinoids,* or sea lilies; and the *Holothuroids,* or sea cucumbers. The various colors shown by these interesting

animals have been described by many zoologists. In general the colors of the echinoderms resemble very closely those of the Crustacea. From the standpoint of the pigments proper the same red and yellow carotinoid-like pigments found in Crustacea appear to be the cause of like colorations, and, in addition, blue and green lipochromogens are also encountered. From observations cited in Miss Newbigin's Monograph (1898) the blue and green colors are more common in species found in shallow water than in the deep-sea forms, where red colors predominate.

Merejowsky (1881) first called attention to the properties, later ascribed to lipochromes, of the red pigment in echinoderms, and cited twenty or more species, representing the various orders, in which it occurred. He used the name zoonerythrine for the pigment, and later (1883) reaffirmed his previous observations, especially the presence of the pigment in the sea cucumber *Holothuria tubulosa,* which had been denied by Krukenberg. The observations on the chromolipoids in the Holothuroids have, in fact, been contradictory. Krukenberg (1882j) called the skin pigment of *Holothuria Poli* uranidine, but later (1882k) stated that "rhodophane" is present in an especially pure condition in the ovaries and blood vessels of this species, while MacMunn (1890) found no lipochromes in this species, but reported a "rhodophane-like lipochrome" in the blood and "liver" of other species, namely *Holothuria nigra* and *H. Ocnius brunneus.* The single absorption band of this pigment in ether was placed by MacMunn at 507-471$\mu\mu$.

It is evident from the observations of Krukenberg (1882k) and MacMunn (1890) that in the star-fishes, at least, chromolipoids showing two-banded spectra and more nearly resembling true carotinoids predominate over the red pigment showing only one band. Krukenberg described such a pigment in the skin and "liver" of the species *Astrospecten aurantiacus* and *Asteracanthion glacialis,* under the name orangin (on account of its color), and MacMunn described similar carotinoid-like pigments in the orange-colored ovaries of *Asterina gibbosa.* These pigments may be xanthophyll, judging from the absorption spectra described by Krukenberg. It is evident, however, that carotin may be the cause of the red, two-banded pigment found by MacMunn in the integument of *Goniaster equestris, Cribella oculata* and *Solaster papposa.* The red ovaries of the *Cribella* species contain the same pigment.

Of the other forms of echinoderms, no special examinations of pig-

ments seem to have been made for the brittle-stars or sea-urchins with the exception of Merejowsky's zoonerythrine-containing species. MacMunn (1890) reported a yellow lipochrome in the Crinoid, *Antedon rosacea,* but attempts to ascertain the character of old extracts from other species of sea lilies were unsatisfactory, as might be expected.

The echinoderms, like the Crustacea, may also contain various lipochromogens. Merejowsky (1883) described a red echinastrine, a green astroviridine, a gray astrogriseine and a violet astroviolettine, each soluble in water and readily going over into "zoonerythrine" like his velelline, described in a preceding paragraph. He also described a brown ophiurine in species of brittle-stars. Ultra-microscopic and ultra-filtration studies on solutions of these and the crustacean "lipochromogens" would throw some light on whether colloidal phenomena are involved, as was suggested above.

Carotinoids in Molluscs

One does not ordinarily associate carotinoid-like colors with these animals among which are represented the various species of oysters, mussels, snails and octopus. Merejowsky (1881, 1883), however, has designated a number of species of gastropods (snails) and conchiæ among the "zoonerythrine" containing animals. It is not stated, however, whether the pigment is in the shells, or in the animals themselves. More specific is the statement of Krukenberg (1882f) that the liver of the gastropod *Helix pomatia* sometimes contains a yellow lipochrome showing two absorption bands, one over F and the other between F and G. MacMunn (1883, 1885a) failed to find such a pigment in the liver of this species as well as a number of other gastropods, finding only "enterochlorophyll," a pigment showing the absorption bands of chlorophyll in the red and green parts of the spectrum, which MacMunn held to be of animal origin. A different result was obtained in the examination of the liver of the mussel *Mytilus edulis,* in which a "lutein" pigment, showing an absorption band at F and one between F and G was found in addition to the "enterochlorophyll." In a more recent study of the pigments of mollusc livers by Dastre (1899) there is described besides the "chlorophylloid" (compare with MacMunn's enterochlorophyll) a pigment called cholechrome, which is stated to be intermediate between bilirubin and lipochrome. Cholechrome, uncontaminated with chloro-

phylloid, is stated to be the liver pigment of Crustacea and other arthropods (spiders, insects).

It seems to be apparent from even these meager studies that the digestive organ, at least, of molluscs may contain a pigment which may be either carotin or xanthophyll or a modification of one of the carotinoids.

Carotinoid in Worms

Miss Newbigin (1898) has given an excellent summary of the brilliant colors, both pigmental and structural, shown by the various species of worms. It is evident that many types of pigments are present. Carotinoïd-like pigments, however, are not entirely absent, if one is to judge from the observations of the older investigators. These observations, unfortunately, have been confined to only a few species so that it is not possible to decide how widely distributed these chromolipoids may be among the worms.

Krukenberg (1882h) found a rhodophane-like lipochrome in the pure uncontaminated digestive juice of *Siphonostoma diplochaitos.* He (1882i) has also described a lipochrome in the cuticular skeleton of the Polyzoa, *Bugula neritina,* whose spectrum is identical with that of carotin. The pigment is not, however, the chief one of this species. According to MacMunn (1890) the orange-red color of two other species of this class of worms, namely, *Lepralia foliacea* and *Flustra foliacea,* is due to a rhodophane-like lipochrome.

Among the Chaetopods, or segmented bristle worms, MacMunn (1890) found several species among the Polychaetes which apparently contain carotinoids. In *Arenicola piscatorium,* a black worm, the intestine was found to be covered with an orange-colored glandular tissue. The pigment could be extracted with the fat solvents and alcohol, and the extracts showed two, possibly three bands in the green and blue. The integument was found to contain the same pigment masked by melanin. In a *Terebella* species the tentacles and integument contained a lipochrome showing two absorption bands. A like pigment was found in the integument of the species *Cirratulus tentaculatus* and *C. cirratus. Nereis virens,* the common clam worm of the northern seas, contained it also, but in smaller quantity. In *Polynoe spinifera* most parts of the worm contained the same lipochrome.

These observations, while brief, point very strongly to the presence of carotinoids in worms. Before passing to the sponges in which

carotinoid-like pigments appear to be widely distributed it might be mentioned that there is no definite evidence that the Coelenterates (sea-anemones, corals, jelly-fish and related animals) contain carotinoids, notwithstanding the brilliant colorations which they exhibit. It is true that Merejowsky (1881) listed numerous species containing tetronerythrine, but Krukenberg (1882g) disproved this for *Gorgonia verrucosa.* MacMunn (1890), however, mentioned a lipochrome resembling rhodophane or xanthophane in the red polyp head of the species *Tubularia indivisa.* Further study is needed of the pigments in this group of animals.

Carotinoids in Sponges

The Porifera, or sponges, when fresh show a variety of colors from red to green, some of which are quite brilliant, but others dull. The yellow, orange and red colors have been found to be due almost exclusively to lipochromes, in the broad sense. There is little doubt that the yellow and orange pigments are true carotinoids. The red pigment, however, appears to belong to the carotin-like group of the same color which is so widely distributed among the lower forms of animal life.

Krukenberg (1880) first discovered the lipochrome nature of the yellow and red pigments of sponges. It was not until he repeated (1882l) his first observations, however, and purified his extracts by saponification that a satisfactory separation of the various pigments was obtained. The technic employed was essentially that used by Kühne in his chromophane studies. The sponges were extracted with alcohol, the extract saponified and the soap salted out with NaCl. This material was then shaken with petroleum ether until no more pigment was extracted. A similar treatment with ether followed, and if any pigment remained the soap was treated with acetic acid and the liberated pigment taken up with CS_2. It is readily seen that this method would not lead to a separation of carotin and xanthophyll. It did serve, however, to separate distinctly carotinoid-like pigments in most cases from the rhodophane-like chromolipoids showing only a single absorption band. In general the petroleum ether extracts showed two absorption bands, and the residues left on evaporation gave the characteristic color reactions with concentrated acids. The bands pictured by Krukenberg in some cases indicate carotin, such

as in the sponges *Hircinia spinosula, Suberites flavus, Tedania Muggiana* and *Suberites massa,* while in others, namely, *Papillina suberea* and *Tethya Lyncureum,* xanthophyll is indicated. The ether extract of the soap, after the petroleum ether treatment, in most cases showed only a single absorption band, from which Krukenberg drew the conclusion as to the presence of a rhodophane-like pigment. Pigment of this character is apparently not present in all sponges. For example, Krukenberg found only carotinoid-like pigments in *Suberites flavus* and *Pipillina suberea.* Other sponges which contain pigment showing two-banded spectra, according to Krukenberg, are *Reniera aquaeductus, Cocospongia, Chondrosia reniformis, Aplysina aerophoba,* and *Suberites domuncula.*

MacMunn (1888) reported spectroscopic studies of the lipochromes of a number of additional species of Porifera, which throw still further light on the widespread occurrence of carotinoid-like pigments in the sponges. His method was to examine the alcoholic extracts of the sponges for absorption bands and then shake the alcoholic solution with CS_2 and repeat his observations on the CS_2 solutions. Neither solution would be expected to show the character of the carotinoids present inasmuch as alcohol extracts both classes of pigments from tissue, and also since xanthophylls are partly epiphasic between alcohol and carbon disulfide. However, if chromolipoid pigments remained in the alcohol after the carbon disulfide extraction, this fact would indicate the presence of xanthophylls.

Almost all of MacMunn's observations of absorption bands of the lipochromes in both alcohol and carbon disulfide show the carotinoid nature of the lipochromes. In general they favor carotin rather than one of the xanthophylls. In three species, namely, *Halma Bucklandi, Halichondria albescens* and *Leuconia Gossei,* one lipochrome was found showing only a single absorption band. The species *Halichondria incrustans* and *Halichondria seriata* may contain both carotin and xanthophylls since the alcohol remaining after the carbon disulfide extraction still showed carotinoid absorption bands. On the other hand, carotin alone may be the chromolipoid in the species *Halichondria caruncula* and *Halichondria rosea,* whose alcohol extracts were left practically colorless by the carbon disulfide. No information of a similar nature is given for the species *Halichondria panicea, Hymeniacidon albescens, Grantia coriacea, Halichondria sanguinea* and *Pachymatisma Johnstonia,* comprising the remaining species examined.

Summary

Carotinoids are abundantly present in the invertebrates but they cannot be said to predominate even among the pigments of yellow to red color. As one descends the scale of animal forms non-carotinoid pigments of yellow to red hues are encountered more and more frequently. The simpler digestive apparatus of the lower animals does not seem to insure a more abundant distribution of biologically derived pigments.

The orders of insects in which carotinoids occur are butterflies, bugs, beetles and locusts (grasshoppers). In the butterflies it is the larvæ and pupae which contain carotinoids, not the butterflies themselves. Although the chromolipoid, present chiefly in the haemolymph (blood) of the larvae and pupae, and also in the eggs, has been known as "xanthophyll" since the work of Poulton (1885) there is evidence to suggest that the pigment is actually carotin.

Among the bugs, the yellow pigment which can be extracted from green plant lice (*Aphids*) is carotinoid in nature but it is not known whether a single pigment or a mixture is concerned. Carotin itself appears to be the cause of the red color of the tegument in the case of certain other species of bugs.

There can be no doubt that the yellow and orange pigments found in the beetles are often, if not always, carotinoids. The red pigment, however, belongs to the carotin-like pigments which conform to the properties of the so-called carotinins described in previous chapters.

The character of the carotinoids which occur in many locusts and grasshoppers is not known; the subject deserves further study.

Two distinct types of chromolipoids are present in Crustacea, one characterized by its red color, the other by a more yellow hue. All the older investigations of the red pigment agree in showing that it differs in its general properties from the known carotinoids only in exhibiting one spectroscopic absorption band and in forming salts with alkalies and alkaline earths. However, Verne (1920a, b) has recently announced that the pigment is identical in every respect with carotin. All the properties of the yellow pigments so far examined suggest carotin, rather than xanthophyll.

The red crustacean carotinoid appears to exist in the shell of various species as a water-soluble substance of blue, brown, orange or green color, which is instantly transformed into the water-insoluble

red carotinoid by heat, acids, alcohol, ether, etc. The author suggests a colloidal explanation for the hitherto inexplicable relations between these apparently water-soluble chromogens and the red pigment.

The same red and yellow carotinoid-like pigments found in Crustacea appear to be the cause of like colorations among the echinoderms (starfish, brittle-stars, sea-urchins, etc.). In addition, red, green, blue and violet lipochromogens are also encountered.

The shells of snails apparently may be colored by the red carotinoid-like pigment already described, and the digestive organs of these animals as well as some molluscs may contain a pigment which is either carotin or xanthophyll or a modification of one of these carotinoids.

The brilliant colors encountered among marine and fresh water worms are due in part to carotin or related pigments. Similar colors among the sea-anemones, corals, jelly-fish and related animals, however, do not appear to involve the carotinoids.

The yellow, orange and red colors of sponges have been found to be due almost exclusively to lipochromes in the broad sense. The yellow and orange pigments are undoubtedly true carotinoids, and the red pigment is the carotin-like substance, showing one absorption band, which is so widely distributed among lower forms of animal life. The presence of both carotin and xanthophylls is indicated among the true carotinoids.

Chapter VI

Chemical Relations between Plant and Animal Carotinoids

It is a chemical axiom, so to speak, that the final proof of the identity of like chemical compounds must be furnished by a chemical analysis of the purified substances, together with complete correspondence in all known chemical and physical properties. It has been stated repeatedly in the preceding chapters that evidence has been presented which shows the character of the chemical relationship between plant and animal carotinoids. It is desired to consider this evidence in more detail in this chapter.

Egg yolk xanthophyll. It is not necessary to present again the observations showing the close relationship between the pigment of the yolk of the hen's egg and the plant chromolipoids, which was known to numerous workers through the macroscopic examination of the pigment. Reference may be made, however, to the spectroscopic studies of Schunck (1903) who first showed the correspondence between the egg yolk pigment and one of the groups of plant carotinoids. Schunk's results, secured largely by a photographic study of the absorption spectra of the pigments separated by inadequate, and unfortunately by inaccurate means, were obtained before carotin and xanthophylls were established as chemical entities. This method was described in Chapter II. It could not have insured the freedom of the "xanthophylls" from admixture with carotin. Nevertheless Schunck was careful to distinguish between xanthophylls and "chrysophyll," which he recognized as probably identical with carotin. The spectrophotographs show this very clearly so that the spectroscopic relations between one of Schunck's flower xanthophylls (his so-called L. xanthophyll) and the egg yolk pigment rightly deserve credit for being the first to show the xanthophyll character of this animal chromolipoid.

Crystals of egg yolk pigment are stated by Willstätter and Escher (1912) to have been observed first by Kühne (1882). It was stated

in Chapter III that Kühne (1878) had previously decided, partly on spectroscopic grounds, that the egg yolk pigment could not be identical with the so-called lutein in the corpus luteum. It remained, however, for Willstätter and Escher to attempt the isolation of the pigment in sufficient quantity for chemical analysis. This proved to be a rather difficult task and involved working with large quantities of material. Starting with the yolks of 6,000 eggs, which weighed 110 kg., only 4 grams of crude crystalline pigment was obtained. The method of isolation will be described in Chapter VIII.

The crude egg yolk pigment was purified first by repeated crystallization from hot methyl alcohol. About 250 cc. of boiling alcohol were required to dissolve 0.25 grams of the crude product, from which approximately 0.16 grams of crystals came down on standing for some hours. It is stated that it was also found possible to obtain crystals showing a constant melting point by dissolving the crude crystals in carbon disulfide and recrystallizing from this solvent. The chemical studies on the purified substance showed the following average results. For comparison similar data are shown for the plant xanthophyll isolated by Willstätter and Mieg (1907).

		Plant xanthophyll	*Egg yolk xanthophyll*
CH_3OH of crystallization, calculated for $C_{40}H_{56}O_2$, CH_3OH .... = per cent		5.33	5.33
CH_3OH found ....per cent		4.76	4.35
Molecular weight in $CHCl_3$, ebulloscopic method, calculated for $C_{40}H_{56}O_2$............		568.4	568.4
Molecular weight found.......................		512.	640.
Elementary analysis; percentage composition required for formula $C_{40}H_{56}O_2$...............	C =	84.44	84.44
	H =	9.93	9.93
Elementary analysis found....................	C =	84.22	83.58
	H =	9.92	10.13
Melting-point (corrected).....................		173.5—174.5°	195—196°C.

An examination of these data shows that the plant xanthophyll analyzed by Willstätter and Mieg showed excellent agreement, at least in chemical composition, with the theoretical values. The results of the analyses of the egg yolk pigment, however, can only be regarded as approximations, at best. The molecular weight determination is also very unsatisfactory. Willstätter and Escher explain the low figure for the content of methyl alcohol of crystallization on the grounds of a possible oxidation of the pigment during the process of removal of the alcohol, which required a period of about 10 days over phosphorus pentoxide. The explanation does not seem entirely satisfactory, however, in view of the statement that this process took

place in a high vacuum. No similar explanation is offered for the divergence of the molecular weight and elementary composition determinations from the theoretical values. It is not apparent from the description given that any less care was taken in purifying the crystals for these analyses than was taken by Willstätter and Mieg in preparing the plant xanthophyll for a like purpose.

When these results are viewed from a strictly chemical standpoint it is difficult to escape the conclusion that the purest preparations were not free from admixture with a substance of high molecular weight which is lower in carbon and higher in hydrogen than the xanthophyll. This conclusion is supported by Willstätter and Escher's own statement that the 4 grams of crude pigment contained a large proportion of wax-like material which had solubility properties similar to the pigment. On the other hand, it is hardly possible that the high melting point of the egg yolk pigment in comparison with the plant xanthophyll, which led Willstätter and Escher to call the pigment xanthophyll "b," was due to the impossibility of removing the unknown impurity. The presence of a wax of high molecular weight would undoubtedly alter the melting point of the pure pigment but would lower, rather than raise it. There is certainly no evidence that the preparations used for the melting point determinations were of any higher purity than those used for the elementary analyses.

The other chemical properties of the egg yolk xanthophyll leave no doubt as to its identity in these respects with the plant xanthophyll. The actual solubility of the two pigments in various solvents is identical. The crystalline form from various solvents agrees perfectly. Both pigments form a violet colored crystalline iodide (showing that Kühne's failure to obtain the blue iodine lipochrome reaction was not due to any peculiar characteristic of the pigment). The phase test applied to the purified pigment shows complete correspondence with the xanthophyll group of carotinoids, the pigment being practically quantitatively hypophasic between petroleum ether and 80-90 per cent ethyl or methyl alcohol. Finally, the spectroscopic absorption bands of the two pigments are identical in every respect when measured under the same conditions.

As pointed out in Chapter IV there is also a biological basis for rejecting Willstätter and Escher's conclusion that the egg yolk xanthophyll is an isomer of plant xanthophyll, unless it be assumed that the plant xanthophyll from which the egg yolk pigment is derived is

modified in the animal body. The hypothesis was also advanced that the egg yolk xanthophyll may be an individual member of the xanthophyll group of carotinoids, which the digestive and assimilative organs of the hen have the ability to select from the group of carotinoids presented to them in the food. Considered solely from a chemical basis, however, it is indeed an extraordinarily closely related isomer which shows such complete correspondence in all its other properties, both chemical and physical, with the single exception of the melting point. Lycopin, the red isomer of carotin, found especially in the tomato, has the same melting point and chemical composition as carotin, but differs from it in a number of chemical properties, such as color, absorption spectrum, solubilities, etc. Therefore, from this point of view, also, it seems unlikely that the alleged isomerism of the egg yolk xanthophyll actually exists.

Serono and Palozzi (1911) claimed to have isolated the lutein of egg yolk by a very different method. Egg yolk was extracted with 95 per cent alcohol, the extract evaporated in vacuum, and the residue treated with acetone. This extract is stated to have contained mostly "lutein," a little cholesterol and traces of lecithin. The lutein was obtained in crystalline form from this solution by precipitation from boiling acetone. The crystals thus secured are described as white to pale yellow radiating clusters with a few crystalline lamella with a blue fluorescence, which turn yellow almost instantly in the air and become more and more colored until a deep red is reached. The analyses of this lutein indicated a mixture of cholesterol, fat and cholesterol esters of oleic and palmitic acids. Egg yolk is stated to contain about 4.04 to 4.17 per cent of this pigment.

With the above observations as a basis it is not surprising that Serono (1912) vigorously attacked Willstätter and Escher's work showing the xanthophyll nature of the egg yolk pigment. It is obvious, of course, that Serono's lutein cannot be the true egg yolk pigment. At the same time the poor correspondence of the egg yolk xanthopyhll with the plant pigment with respect to the melting point and elementary composition naturally offered splendid points of attack. Accordingly Serono's assertion is quite incontrovertible that the carbon content found for the egg yolk pigment by Willstätter and Escher (83.58 per cent) corresponds better with the carbon content of an oleic acid ester of cholesterol (83.33 per cent) than it does with the carbon content of carotin dioxide (84.44 per cent). Although one would hardly be tempted to accept Serono's conclusions regarding a

cholesterol ester constitution for the egg yolk pigment, it must be admitted, nevertheless, that if it were not for the complete correspondence of the general chemical properties of the egg yolk pigment with plant xanthophyll, it would not be possible to decide on chemical grounds, from the evidence submitted, that the two substances are identical or even isomers.

Corpus luteum carotin. It was pointed out in a previous chapter that the pigment in the corpus luteum tissue on the ovaries of the cow was one of the first to attract the attention of those interested in discovering the nature of animal pigments. It was certainly the first animal carotinoid to be obtained in crystalline form if one is to accept the early work of Piccolo and Lieben (1866) and Holm (1867). Although a number of later workers described the chemical properties of the pigment, as was shown in Chapter IV, Willstätter and Escher (1912) are to be credited with first stating the exact chemical relationship of the corpus luteum pigment to plant carotin. Their work was not the first to show a connection between animal coloring matter and plant carotin; nor were they the first to use the name carotin for an animal pigment. It will be recalled that Zopf used the name carotin in the form of "eucarotin," "di-carotin," "monocarotin," "carotinin," etc., for various animal pigments studied by him. He certainly recognized the relation between the vegetable and animal "carotins," to which he gave the various designations mentioned. It is apparent, however, that the name itself was a collective name in Zopf's mind and that both carotin and xanthophylls, in the sense we now know them, were included. For example, Gerlach (1892) working under Zopf's direction, refers to the egg yolk pigment as di-carotin. On the other hand Physalix (1894) actually had Arnaud's carrot carotin in mind, as his paper shows, when he assigned the name carotin to the pigment which he isolated from the red tegument of the bug *Pyrrhocoris apterus*. The insect carotin was not, however, analyzed.

It was no doubt the development of the Kraus method for separating the plant carotinoids into specific carotin and xanthophyll groups, in which Tswett and Willstätter played a dominant part, that led Willstätter to examine certain of the common animal lipochromes by this procedure. The discovery of the xanthophyll character of the egg yolk pigment by this means naturally prompted the study of the corpus luteum pigment, since the observations of the early workers had shown that it could be obtained in crystalline form without dif-

ficulty. Willstätter and Escher announced their discovery of the carotin nature of the corpus luteum pigment in connection with their studies on egg yolk xanthophyll. The details of the corpus luteum work were later published by Escher (1913).

The relatively small size of corpora lutea tissue naturally required the collection of ovaries on a large scale. It was fortunately found possible to preserve the ovaries for many months under 60 per cent alcohol without impairment of the pigment (preservation in dilute formalin prevented the isolation of crystals), thus making it feasible to collect the material in large slaughter houses over an extended period of time. After 146 kgs. (about 10,000 ovaries) has been collected from cows and sheep, the tissue was hashed in 10 kg. lots, further dehydrated with 95 per cent alcohol, and then shaken in the cold with petroleum ether (b. p. 50°-70° C.) for several hours. This effected an almost complete extraction of pigment. This extract (about 3 liters for each 10 kg. of ovaries) was then washed seven successive times with one-sixth volume of 90 per cent methyl alcohol, the alcohol removed by washing four times with one-third volume of water, the extract freed from water by shaking with anhydrous sodium sulfate, filtered and concentrated to a syrup in vacuum. On adding six to ten volumes of absolute ethyl alcohol and cooling in an ice-salt bath nearly all fatty impurities were precipitated. These were separated quickly by means of a cloth filter, for in a short time crystallization of the pigment began and continued for several hours, at ice-box temperature. Some very large (1-2 mm. long), beautiful crystals were obtained. Purification was secured by filtering and washing with a mixture of equal parts petroleum ether and absolute alcohol. Only 0.45 grams of pigment in all were secured in this way from the 146 kgs. of ovaries.

The crude ovarian pigment proved to be remarkably pure as judged from the microscopic examination and melting point of the crystals. For the elementary analyses, however, the pigment was recrystallized first from alcohol, again by addition of an excess of absolute alcohol to a concentrated carbon disulfide solution, and finally from pure petroleum ether (sp. g. 0.64-.66). The elementary composition showed the pigment to be a hydrocarbon. The average of four closely concordant analyses carried out for Escher by Fisher and Sonnenfeld under Willstätter's direction, using Preyl's micro-method, showed 89.55 per cent carbon and 10.66 per cent hydrogen. This corresponds even more closely to the calculated composition of the compound $C_{40}H_{56}$

than Willstätter and Mieg obtained in their analyses of the carotin from either green leaves or carrots. Although Escher was unable to carry out any molecular weight determinations because of lack of material, there can be no doubt from these data that the ovarian carotin is identical in composition with the carotin of vegetable origin.

Escher's observations of the melting-point of ovarian, carrot and leaf carotins are of interest. By heating crystalline preparations from each source together in the same bath using a shortened thermometer he found that they all melted at exactly the same temperature, but that this temperature was about 175° C. (corrected). This temperature is appreciably higher than the figure reported by Willstätter and Mieg for the carotins of plant origin, namely, 167.5°-168° C., which corresponded with the earliest observations of Arnaud, Kohl and others. Escher explained his results on the grounds that considerable variation in melting point can be obtained by modifying the method of heating, so that in reality the melting point of crystals of different carotinoids should be determined under comparative conditions to secure comparable results. These results may indicate, therefore, that the slight difference in the melting point of carotin and xanthophyll crystals may not be of great importance in distinguishing the two forms of carotinoid.

The general properties of the ovarian carotin also correspond exactly with the carotin from carrots and leaves. The crystalline form; solubility of the crystals in various solvents; response to the phase test (being practically quantitatively epiphasic between petroleum ether and 80-90 per cent alcohol); position of the absorption bands; formation of a crystalline tri-iodide ($C_{40}H_{56}I_3$) in carbon disulfide solution, melting at 133.5°-135° C., and showing 40.6 per cent iodine (calculated value 41.5 per cent); and its ready oxidation to a grayish-yellow powder, soluble in alcohol and containing 36 per cent oxygen, all corresponded exactly with the properties of carotin from leaves and carrots. There can be no doubt whatever that the carotin from the corpus luteum of the cow is identical with the carotin so widely distributed in the vegetable kingdom.

Crustacean carotin. As stated in Chapter V in connection with the chromolipoids of Crustacea, Verne (1920) has recently stated that the red pigment in the hypodermis of the higher Crustacea, such as lobsters, crabs and crayfish, is identical with carrot carotin. The analytical data are not presented. In support of his conclusion, however, Verne makes, in substance, the following assertions. The pigment

was isolated by the methods of Arnaud and Escher. The crystals melted at 168° C., and gave an iodide of definite composition. The elementary analyses of a large number of samples obtained from Crustacea of various kinds showed the pigment to be a hydrocarbon in which the ratio of $C:H = 5:7$. The molecular weight determination performed on the iodides by the ebulloscopic method showed that the carotin of Crustacea is the same as that of vegetables, with the formula $C_{40}H_{56}$. Among its most significant reactions was the formation of a violet-brown iodide. The pigment exhibited the same spectroscopic absorption bands as vegetable carotin. It was not attacked by alkalis and oxidized with great ease.

These statements are certainly sufficient to establish the carotin identity of the crustacean pigment. It is to be hoped, however, that the details of this study will soon be made available. Certain of the points mentioned in connection with the general properties of the pigment are quite at variance with the findings of numerous previous investigators who were presumably studying the same crustacean pigment. Either Verne was working with a different pigment, or the methods used by previous investigators were decidedly at fault. It is important that these divergencies be explained.

Summary

A chemical relationship between an animal chromolipoid and a specific plant carotinoid was shown for the first time by Schunck's (1903) comparative spectroscopic studies of flower "xanthophylls" and the yellow pigment of the egg yolk and blood serum of fowls.

Chemical studies of crystals of egg yolk pigment by Willstätter and Escher (1912) showed a complete correspondence with plant xanthophyll in all properties except melting point, but the results of the elementary analyses and molecular weight determinations of the egg yolk pigment can only be regarded as approximations to the theoretical values for a substance with the formula $C_{40}H_{56}O_2$. A consideration of these divergencies in the light of the biological relationship between egg yolk pigment and plant xanthophyll leads to doubts regarding the alleged isomerism of the two pigments.

A possible chemical relationship between certain animal chromolipoids and plant carotin was recognized by several workers before Escher (1913) definitely established the chemical identity of the corpus luteum pigment (of the cow) with plant carotin.

Escher's study of the comparative melting points of ovarian, carrot and leaf carotin shows that slight differences between the melting point of different carotinoids are not significant unless the determinations are carried out simultaneously.

The recent work of Verne (1920) on the identity of the red crustacean chromolipoid with plant carotin throws doubt on the results of numerous previous workers which indicate that the pigments are related but not identical.

Chapter VII

Biological Relations between Plant and Animal Carotinoids

The origin of color in animals, when considered by and large, has been, until recently, practically an unexplored field. So far as the colors which might be due to carotinoids or related pigments are concerned no systematic study of their possible biological relationship to plant pigments of similar color was undertaken previous to the investigations by Palmer and Eckles, published in 1914. A few close students of the subject have, indeed, suggested such a relationship in isolated cases, which are mentioned below, and there are also certain isolated observations which support the idea. It is a striking fact, however, that so little experimental work had been done in this field that even the chemical identification of certain of the animal lipochromes with plant carotinoids by Willstätter and Escher (1912) and by Escher (1913) apparently raised no query in their minds as to their possible origin from the plant pigments. For example, Escher, in concluding the paper on corpus luteum carotin remarks, "What this unsaturated terpene hydrocarbon, carotin, which is so widely distributed in the plant world, is doing in such an important gland as the corpus luteum, can not even be conjectured at present. —there is nothing in the literature to establish whether it is a substance produced from one of the specific gland cells, or is only a pigment which has been resorbed by the cells from the blood extravates." It is clear, also, that Escher saw no biological relationship between the xanthophyll of the egg yolk and plant xanthophylls, for he expresses the view that, "the oxygen-containing lutein ($C_{40}H_{56}O_2$) in the yolk of eggs plays the part of an atavistic plant respiratory pigment for the formation of hemoglobin in the embryo."

It is true that when Fischer and Röse (1913) isolated carotin from the gall stones of cattle they were unable to agree with Escher's conclusion and suggested, rather, that the carotin in the cow's body probably comes from the food. In reality, however, Escher's general

view that the animal lipochromes are of animal origin coincides with Krukenberg's (1886) conclusion in the summary of his extensive chromolipoid studies, where it is stated that, "they (i.e., the lipochromes) originate in most cases from fat-like substances." A more specific instance of the same general conclusion reached by one of the foremost earlier workers on animal lipochromes is found in the paper by Zopf (1893a) describing the yellow carotin-like pigment in the little fresh-water crustacean, *Diaptomus bacillifer.* Zopf states, "I could mention an objection which could be raised against the two-banded yellow carotin found in these Crustacea. One could say that it is not produced by the organs of the crab but perhaps comes from the chlorophyll-containing algae which serve as their food, and which contain a two-banded yellow carotin. However, this can not be true because the animals with which I worked were preserved in alcohol and no trace of chlorophyll was extracted which would have been the case had algæ been present in their digestive tract."—"The antennæ of *Diaptomus Castor Jurine* is colored exclusively by diaptomin while the body cavity contains fat masses of purest yellow. I believe that the yellow carotin is produced by these animals just like the diaptomin." It is clear from these citations that Zopf saw no evidence of a biological relationship between plant and animal carotinoids.

On the other hand Poulton (1885) believed that yellow pigment in caterpillars was derived from the "xanthophyll" (carotinoids) of the food and the green color from chlorophyll. Poulton later (1893) submitted his experimental proof of the derivation of chlorophyll by the caterpillar from its food, an experiment which is classic so far as the demonstration of derived pigments in animal colorations is concerned. Goode (1890) made a particularly interesting observation, which can hardly be classed as an experiment, but which verifies the probability of a biological relationship between plant and animal pigments in another species. To quote directly from his paper: "On certain ledges along the New England coast the rocks are covered with dense growths of scarlet and crimson seaweeds. The codfish, the cunner, the sea raven, the rock-eel, and the wrymouth, which inhabit these brilliant groves, are all colored to match their surroundings; the cod, which has naturally the lightest color, being most brilliant in its scarlet hues, while the others, whose skins have a larger original supply of black, have deeper tints of dark red and ruddy brown."—"It has occurred to me that the material for the pigmentary secretion is probably derived indirectly from the algae, for, though the species

referred to do not feed upon these plants, they devour in immense quantities the invertebrate animals inhabiting the same region, many of which are likewise deeply tinged with red. Possibly the blacks and greens which prevail among the inhabitants of other colored bottoms are likewise dependent upon coloring matter which is absorbed with the food. Günther believes that the pink color in the flesh of the salmon is due to the absorption of the coloring matter of the crustaceans they feed upon."

Miss Newbigin, both in her papers (1898) and her Monograph (1898) takes a somewhat intermediate position on the question of derived animal pigments. Regarding insects, she accepts Poulton's results but qualifies them by stating that, "At the same time there is no apparent reason why insects should not themselves produce lipochromes, and why such lipochromes should not occur in the cuticle as in the Crustacea." With reference to the carotinoids in birds, she states with more conviction, that "although there are several instances described of birds whose colors can be heightened or altered by the employment of special kinds of food, there is at present no reason to doubt that under ordinary circumstances the lipochromes of birds are self-produced and not derived."

Miss Newbigin gives a more extensive presentation of her views on this subject in her paper on salmon pigments (1898). It will be recalled that she found a red lipochrome and a yellow pigment which she could not identify as a true lipochrome in the muscle and ovaries of this fish. In discussing these findings she states, "The most obvious explanation is that the pigments of the salmon are derived directly from its food. . . . At first sight the suggestion has much to recommend it. . . . There are, however, some difficulties in the way of the acceptance of this suggestion. In the first place, the salmon seems to feed chiefly on haddock, herring, and similar fish, so that the transfer of pigment can hardly be direct. The herring, however, feeds habitually on small Crustacea, so that it might be said that the pigments of the salmon are obtained indirectly from herring which forms its food." Miss Newbigin, however, was unable to find the red pigment in herring, but did find a small amount of the yellow pigment in the viscera and muscles. In support of the general proposition of animal lipochromes being derived from the food Poulton's experiments are first cited and then the fact that, "it is not uncommon to find the fat of sheep (?) [1] and cows dyed a deep yellow color. According to some

[1] Question by author.

authorities, this occurs quite sporadically without known cause, while according to others special foods, notably maize, are the important agents." Miss Newbigin then states that she secured the lipochrome color reactions with the maize pigment but not with the pigment from yellow fat. "In other respects, in tint, in solubility, and so on, the pigments closely resemble each other. This fact, taken in combination with Mr. Poulton's experiments, seems to me at least to prove the possibility of the transference of these pigments from one organism to another, and therefore to suggest such an origin for the yellow pigment of the salmon."

On further consideration Miss Newbigin concluded that the derivation of yellow pigment from the food could not be very general, otherwise pigmented fat would be universally found in herbivorous animals, which Miss Newbigin knew is not the case. Her explanation of the phenomenon which she believed to be peculiar to caterpillars, salmon and domesticated cattle was that they ingested more colored fat in their food than they required with the result that, "fat colored with the pigment in more or less modified condition is deposited in certain of the tissues."

Miss Newbigin's views are quoted at some length because they not only have a direct bearing on some of the experiments of Palmer and Eckles, but they are the most definite of any of the earlier views on the subject of a possible general origin of animal lipochromes from plants. Although Miss Newbigin decided against any such general relationship between plant and animal pigments the writer has found several isolated observations which support the idea when viewed in the light of our present knowledge. These may be mentioned briefly.

Schneider (1799) observed a great many years ago that the toad, *Bufo viridis*, loses its green color on wintering in the earth. Von Wittich (1854) noted that the frog, *Rana esculenta*, took on a grayish-brown instead of its usual green color after long fasting and that this was accompanied by a disappearance of the yellow cells in the tegument. The disappearance of the lipochromes from the skin of fasting frogs was confirmed by Hering and Hoyer (1869), who also noted its slow reappearance when the animals were given their usual food. Bate and Westwood (1869) stated that the color of *Idotea* is influenced by the food, in that animals which eat *Fucus* are dark or black, while those which eat green algæ are green. This statement, however, has been denied by Möbius (1873) and Matzdorff (1883). Beddard (1892) cited the observation of Eisig that certain marine worms be-

come colored by the lipochromes of the sponge upon which they live as a parasite. Dastre (1899) noticed that he could suppress a chlorophyll-like pigment which occurs normally in the liver of molluscs by withholding chlorophyll-containing food, and that the pigment was also absent from the liver at the end of the hibernation period. Villard (1903) and Przibram (1906, 1907, 1909) found that leaf lice which are raised on etiolated plants in the dark are mostly pale yellow. Schneider (1908) mentions that he found that the crab-eating perch, *Perca fluviatilis,* takes on the characteristic red color of the crab in various places on its body. He thought that the pigment, which he speaks of as crustaceorubin, took the place of a red pigment which normally colors the fish, but it seems more likely that the normal red pigment of the fish is the carotinoid derived from its food.

A still more striking as well as very recent instance of biological relationship affecting lipochromes is mentioned by Gerould (1921) in connection with a blue-green mutation of the normally grass-green caterpillar *Colias* (*Eurymus*) *Philadice,* the blue mutant lacking the normal lipochrome in its hemolymph, eye, cuticle, etc., and the eggs of the butterfly from the blue mutant being pure white instead of the usual yellow. The biological relationship involving the lipochrome is between the caterpillar and the color of the cocoons spun by the parasite *Apanteles flaviconchæ* which emerges from it. These cocoons are normally yellow from the normal, lipochrome containing grass-green caterpillar, but were pure white from the blue-green mutant which lacked lipochrome. According to Gerould, "Yellow blood in silkworms is closely correlated with the spinning of yellow silk, white blood with white silk. Ude (1919), however, has discovered a strain of yellow stock that spins white silk, although their silk glands are yellow."

All of the above citations support the idea of a relationship between plant and animal pigments and even between pigments among animals, which is more than a mere chemical relationship or identity. An especially striking argument supporting the existence of a general biological relation such as is suggested by these instances is furnished by the fact, pointed out by Beddard (1892), that there is a uniform absence of pigment from cave animals coincident with the absence of chlorophyll from cave plants.

The number of scattered observations supporting this thesis is fairly gratifying. Prior to the work of Palmer and Eckles (1914), however, very few definite experiments were carried out which show the possi-

bility of transferring carotinoid pigment from plants to animals, or which were designed to determine whether any of the normal pigments of animals are merely derived from the food.

The earliest, as well as the most interesting experiment of this kind, so far as carotinoids are concerned, was conducted by Sauermann (1889) who studied the effect of feeding cayenne pepper to birds. He became interested in the problem because of the custom in vogue at that time of coloring the feathers of canary birds by feeding them red pepper. It is stated in the paper that the canary bird dealers who practiced this artificial coloring mixed the cayenne pepper with egg yolk and bread and fed the mixture to the very young birds or to old birds during the molting season, thereby coloring the new feathers a yellow to red color. When Sauermann tried the experiment using the red pods from which the pepper but not the pigment had been extracted with 60 per cent alcohol, the pepper plants had scarcely any effect on the color of the plumage. The same result followed the feeding of the crude pigment which had been extracted with absolute alcohol, but when this extract was dissolved in sunflower oil the results reported by the canary bird dealers were confirmed.

Especially interesting were Sauermann's experiments on feeding the pepper pigment to fowls. In this case the cayenne pepper itself was fed to 12 white Italian fowls, 8 weeks old, the young chickens being fed 25 grams of the pepper night and morning mixed with moistened bread and potatoes. The birds received corn and oats in addition. It is stated that the feet of all the fowls became orange and that the pepper pigment could be extracted from them by soaking them in alcohol for a long time and then extracting with ether. No proof is given for the fact that the pigment extracted in this manner was the pepper pigment. Only two of the 12 fowls showed any effects of the pepper feeding on the feathers. The pigment began to appear on the breast of one hen in about 10 days and the other in about 3 weeks. The first hen eventually developed a red breast and the rest of the body became yellowish red, but the second hen only developed red feathers on the breast, the rest of the body remaining white. Old hens were not influenced in the least by pepper-feeding, even during the molting season. If the pigment which appeared on the feathers of the two young birds was the red pepper pigment, it is difficult to understand why none of the other young birds were affected, or why the old hens did not develop some tint in the new feathers formed during the molt.

Somewhat more convincing were Sauermann's results on feeding the red pepper to laying hens. By feeding a hen 5 grams of the pepper each day the pigment appeared in the third egg laid after the beginning of the pepper feeding, as a thin band of color at the periphery of the yolk. By the time the sixth egg was laid the yolk was entirely pigmented. Two interesting properties were noticed in connection with the yolks colored by the pepper pigment: (1) it was impossible to hard-boil them, (2) ether would not extract all the color from the dried yolks, because a part of the pigment was apparently bound tightly to the protein.

These experiments have a bearing on the biological relationship between plant and animal carotinoids for two reasons. They not only record the first authentic instance in which a plant carotinoid was transferred to an animal under experimental conditions, but are also the only experiments showing the possibility of lycopin occurring in the animal body. It was shown in Chapter II that the evidence indicates that the chief pigment in the ripe fruit of the pepper plant, *Capsicum annum,* is the red carotin isomer, lycopin. Presumably this was the chief pigment in the cayenne pepper which Sauermann fed to his hens, and which appeared in the egg yolks and in the feathers in two of the birds. The evidence, although circumstantial, is strongly in favor of this deduction, and should be submitted to further verification because the result presents the apparent anomaly that lycopin, the isomer of carotin, can be transferred abundantly to the egg yolk of the hen while carotin appears in the yolk only in traces even under the most favorable conditions.

The next experiment was that of Poulton (1893), carried out to verify his previous (1885) hypothesis that the colors of caterpillars are due largely to plant pigments derived from the food. Newly hatched larvæ were placed on three diets: (1) yellow etiolated leaves from the center of a heart of cabbage, (2) white mid-rib of cabbage containing no pigment, and (3) deep green external leaves. All were kept in the dark. The larvæ raised on the green leaves and the etiolated leaves grew normally, those on the etiolated leaves growing far more rapidly than those on the green leaves. Both of these sets of caterpillars developed the normal green and brown colors of the species. The caterpillars on the colorless cabbage did very poorly and only one was raised to adult size. This individual, however, remained colorless throughout the experiment. Some of the group of caterpillars on the colorless food were placed on the etiolated leaves after growing

to a certain size, but these did not develop normal color. It is especially interesting that the larvae on the etiolated leaves, as well as those on the green leaves, should have developed green color. Poulton thought that this indicated that the pigment of the etiolated leaf is closely related chemically to chlorophyll, but since the pigment of the etiolated leaf is now generally regarded as consisting chiefly of carotin, the explanation of the greening of the caterpillars must lie in their failure to inhibit the development of chlorophyll from its precursors in the etiolated leaf. Poulton's experiments have been accepted as having proved that caterpillars derive their green and yellow pigments from their food. In fact, it is generally held at the present time that all phytophagous insects derive their lipochromes and chlorophyll-like pigments from their food, and that the former, at least, are passed on from the larvae to the adults.

Gamble (1910) attempted to determine whether the pigments which develop in the hypodermis of the young crustacean *Hippolyte varians*, is derived from the food. The newly-hatched larvae of this species are colorless with the exception of lines of red pigment on the hypodermis. When the adolescent larvæ are placed among green or red seaweeds, the entire hypodermis will turn green or red in 48 hours. Gamble placed the colorless adolescent Crustacea in the inner chamber of double-walled glass vessels, and put a mass of green or red or brown algæ in the outer chamber and fed the Crustacea various foods, such as etiolated *Laminaria*, red crab meat, colorless crab ovaries, and red crab ovaries. At the end of several days the Crustacea had in most cases developed a color similar to their surroundings rather than like that of their food. The conclusion, therefore, seems justified that the red, green and brown colors in *Hippolyte* are not derived from the food. No observation seems to have been made, however, on the yellow pigment which occurs in the chromatophores of the fully developed Crustacea. Gamble states that true yellow pigment is absent from the chromatophores of the newly hatched larvae.

In addition to the experiments of Sauermann, Poulton and Gamble, there remains to be mentioned that of Dombrowsky (1904) who observed that the milk of a goat was tinted by feeding it carrots, and also that of Moro (1908) who noticed a tinting of the skin of children fed bountifully on carrot soup. The actual transference of carotin from the food to the tissues and secretions of man and the higher animals is at least indicated, although not demonstrated, in these cases.

The writer's attention was attracted to a possible biological rela-

tionship between plant and animal carotinoids in 1912 in order to explain the quantitative variations in the pigmentation of butter fat due to changes in the ration of the cow. A study of the chemical and physical properties of the butter fat pigment had shown it to be identical with carotin, regardless of the extent of the pigmentation of the butter fat from which the pigment was isolated. The more highly tinted fats, however, showed the presence of small amounts of xanthophylls associated with the carotin when the total pigment was examined by means of the phase test or analyzed by means of a Tswett chromatogram. When these facts were viewed in the light of the distribution and amount of carotinoid pigments in the usual dairy cattle foods, and when numerous data on the variations in the color of butter fat under known feeding conditions were interpreted with these facts in mind the conclusion was inevitable that the carotinoids of butter fat are derived from the carotinoids of the food. This conviction was strengthened further by a study of the character of the pigment of the adipose tissue, skin secretions and especially the blood serum of dairy cattle showing that the pigment in each case is chiefly carotin, with which a small amount of xanthophyll is usually associated. The preliminary statement of Willstätter and Escher (1912), published during the course of these studies, that the corpus luteum pigment of the cow is also carotin (an observation which we were able to confirm), lent additional support to the theory of a biological relationship between the lipochromes in cattle and the carotinoids of their ration.

The correctness of this theory was shown by varying the content of carotinoids in the ration of the cow through the proper selection of foods deficient in carotinoids or containing an abundance of these pigments and observing the quantitative variations in the amount of pigment in the blood serum and butter fat. These experiments were supplemented by an examination of the character of the pigment in the blood and butter. In addition two dairy cows of the Jersey breed, whose adipose tissue is normally highly pigmented with carotin, were fattened on rations respectively rich and poor in carotin after a preliminary period of sixty days on straw alone. Some of the data secured in the experiments designed to show the biological relation between the carotin content of the blood serum and milk fat and that of the ration are given in Table 14. Table 15 shows the effect on the color of the adipose tissue in certain parts of the body of Jersey cattle which results when partially starved animals are fattened on rations deficient or rich in carotin.

TABLE 14.—THE RELATION BETWEEN CAROTIN-RICH AND CAROTIN-POOR RATIONS AND THE COLOR OF MILK FAT AND BLOOD SERUM

Breed of Cow	Ration	Butter Fat Yellow	Butter Fat Red	Blood Serum Yellow	Blood Serum Red
	Carotin-poor rations				
Ayrshire	Cottonseed meal and cottonseed hulls	1.3[2]	0.4	3.3[3]	0.5
"	Cottonseed hulls, timothy hay and white corn	1.2	0.4	2.6	1.1
"	Cottonseed meal, cottonseed hulls, timothy hay and yellow corn	2.0	0.5	4.9	1.2
Holstein	Cottonseed hulls, corn stover and cottonseed meal	8.5	1.4	6.0	0.7
"	Cottonseed hulls, corn stover and cottonseed meal	3.0	0.7	7.0	0.8
Ayrshire	Cottonseed hulls, corn stover and cottonseed meal	2.5	0.6	11.0	0.9
Jersey	Cottonseed hulls, corn stover and cottonseed meal	11.0	1.7	10.0	0.9
"	Cottonseed hulls, corn stover and cottonseed meal	5.2	1.2	13.0	1.1
"	Cottonseed hulls, corn stover and cottonseed meal	4.7	1.5	7.5	0.7
	Carotin-rich rations				
Ayrshire	Cottonseed meal, cottonseed hulls, timothy hay, yellow corn and carrots	24.0	1.3	54.0	1.8
"	Mixed grain, green alfalfa hay and fresh pasture grass	16.0	1.1	40.0	1.0
Holstein	Mixed grain, green alfalfa hay and fresh pasture grass	54.0	1.8	48.0	1.1
"	Mixed grain, green alfalfa hay and fresh pasture grass	22.0	1.2	41.0	1.0
Jersey	Mixed grain, green alfalfa hay and fresh pasture grass	64.0	2.0	45.0	1.1
"	Mixed grain, green alfalfa hay and fresh pasture grass	54.0	1.7	57.0	1.8
"	Mixed grain, green alfalfa hay and fresh pasture grass	47.0	1.6	45.0	1.0

TABLE 15.—THE RELATION BETWEEN CAROTIN-POOR AND CAROTIN-RICH RATIONS AND THE COLOR OF THE ADIPOSE TISSUE OF DAIRY CATTLE DEPOSITED DURING THE FEEDING OF THESE RATIONS

	Color[4] of [illegible] T[illegible]			
	Carotin-rich Ration		[illegible] Ration	
Source of Adipose Tissue	Yellow	Red	Yellow	Red
Inside of rib	50.0	2.3	1.4	0.1
Mesentery	47.0	2.1	3.6	0.5
Thoracic cavity	29.0	1.3	8.0	1.0
Around ovaries and uterus	49.0	2.3	2.5	0.3
Attached to omasum	33.0	1.6	24.0	1.7
In pelvic cavity	50.0	2.3	47.0	2.1
Around kidney	54.0	1.6	50.0	2.1
Over last rib	47.0	2.3	50.0	2.1
Over outside chuck	47.0	2.0	47.0	1.8

[2] The color of the butter fat was determined by matching a 1-inch layer of rendered melted fat with the color glasses of the Lovibond tintometer.

[3] The color of the blood serum was determined by matching the extract from 10 cc. of serum in 12.5 cc. volume and 1-inch layer with the color glasses of the Lovibond tintometer.

[4] The color readings were taken on a 1-inch layer of rendered, melted fat, using the Lovibond tintometer.

The experiments demonstrated conclusively that the carotin content of the cow's tissues as well as that secreted in the milk fat is determined by the carotin content of the ration. One of the interesting features of the experiments was the demonstration that this relation is independent of the breed of the cow; just as striking changes in the pigmentation of the milk fat, blood serum and adipose tissue were brought about in the highly colored breeds as in those which are not usually so highly pigmented. So far as the blood serum is concerned the breed appears to have no bearing on the maximum carotin content, as the data in Table 14 show. This fact, together with a certain lack of parallelism between the changes in the carotin content of the blood and corresponding changes in the carotin content of the milk fat indicates that the breed differences involving the color of the milk fat and adipose tissue are determined at the site of the synthesis of the milk fat and adipose tissue. It is not at all improbable that the carotin-albumin complex which carries the carotin in the blood serum plays a prominent part in controlling these differences.

A surprising feature of the experiments was the failure of a xanthophyll-rich cattle food, such as yellow maize, to exert any appreciable influence on the color of butter fat. This is brought out clearly in Table 14. In the experiment reported in that table the ration contained 6 pounds of yellow maize daily. In other experiments reported by Palmer and Eckles (1914a) as much as 12 pounds of yellow maize was fed without effect. These results are contrary to popular opinion (compare Newbigin, quoted above), but are unquestionably explained by the fact that carotin is only a minor fraction of the pigment of yellow maize, the major pigment being xanthophyll, which appears to play very little part in coloring the tissues or fluids of dairy cattle.

A word should perhaps be said regarding the experiment whose results are summarized in Table 15. It is obvious on inspecting these data that only certain parts of the body were affected. This is explained by the fact that the preliminary starvation period of the animals failed to remove appreciable amounts of fats from the outside of the body or from around some of the vital organs. It seems evident that the mesentery and related fats were drawn upon chiefly during the period of partial starvation because it was the fat deposited in these parts during the fattening period that was affected by the carotinoid-deficient ration.

Palmer (1915) carried out similar experiments with fowls. The

results not only confirmed the findings of Willstätter and Escher (1912) as to the xanthophyll character of the major pigment of egg yolk, but demonstrated as well that the same pigment is found in the blood serum and adipose tissue. The results of the earlier studies on the origin of the carotin in cattle naturally suggested that the xanthophyll in the tissues of the fowl and in the yolks of its egg is similarly derived from the xanthophyll of the food. This was demonstrated to be the case in carefully controlled feeding experiments in which a xanthophyll-rich ration (containing an abundance of yellow maize), a carotin-rich ration (containing an abundance of carrots) and a carotinoid-poor ration were fed to laying hens. The yolks of the eggs increased materially in color on the xanthophyll-rich ration, and the blood serum was also rich in pigment, but there was a marked decline in the color of the egg yolks from the hens on the carotin-rich and carotinoid-poor rations which was practically parallel and which was accompanied by almost carotinoid-free blood serum. The experiments showed very clearly, however, that there is not an absolute exclusion of carotin by the hen; the egg yolks, adipose tissue and blood serum always contained a small proportion of the total pigment in the form of carotin, which was clearly somewhat greater in the yolks of the eggs from the carrot-fed hens.

These experiments on the biological relation of the carotinoids of fowls to the carotinoids of the food found complete confirmation in the experiments of Palmer and Kempster (1919 a, b, c) in which a flock of White Leghorn fowls was raised to maturity from the time of hatching on rations so devoid of carotinoids that the mature birds showed only the merest traces of pigment in adipose tissue and no demonstrable amounts in the blood serum or skin and none in the yolks of the eggs laid by the mature hens. Xanthophyll-rich feeds brought about a rapid coloration in all parts of the body and in the egg yolks (except in the case of laying hens when the egg yolks only were colored) while carotin, fed in the form of highly colored (colostrum) butter fat had practically no effect on the color of the bird's tissues.

It was found in connection with the writer's (1914e) milk fat studies that the pigment of human milk fat consists of both carotin and xanthophyll. By analogy with the cattle experiments it was concluded that the lipochromes in the human body are likewise derived from the carotinoids of the diet. Hess and Myers (1919) later demonstrated this to be the case in experiments in which it was shown that

the skin of infants can be colored with carotinoids by feeding diets rich in carotin or xanthophyll, and that this was accompanied by an increased lipochrome content of the blood serum. The latter was identified as carotin in the case of carrot feeding. Hess and Myers state that the skin pigmentation resulted in infants "when the dietary included two oranges a day, or the yolk of one egg in the milk formula for a period of two months, or two ounces of spinach daily for a month," as well as when the equivalent of two tablespoonfuls of fresh carrots was fed each day for a period of four to six weeks. An increase in the carotin content of the blood serum was also noted following a subcutaneous injection of carotin (from carrots) dissolved in olive oil, and the pigment also appeared in the urine. The latter phenomenon was noted also when a concentrated carotin solution in olive oil was given to an infant by mouth.

The general fact that man and the higher mammals and the fowls derive the chromolipoids of their tissues and secretions from the carotinoids of their food is now well established. Van den Bergh,[5] Muller and Broekmeyer (1920), especially, have contributed much valuable data on the variations in the carotinoids in the human body under normal and diseased conditions, as well as contributing observations on the character and extent of the carotinoid pigmentation in various species of animals. The numerous reports of the skin colorations of diabetics, known as Xanthosis, Carotinemia, Lipochromemia, etc., which were cited briefly in Chapter IV, also support and confirm the biological relationship between plant and animal carotinoids, at least for the higher animals. Since this has already been demonstrated to be the case for the phytophagous insects there seems to be no valid reason for rejecting the general thesis that all animal lipochromes are derived from the carotinoids of the food. This conclusion must be accepted for the herbivorous animals of all species, both vertebrates and invertebrates, in which lipochromes are found. When one considers that those animals which prey solely on lower forms of animal life usually, if not always, select their food among species which are herbivorous, then the possibility becomes practicable, if not demon-

[5] In this paper van den Bergh lays claim to an unpublished study carried out by him in 1913 in which he showed that the lipochrome content of the blood serum of men, fowls and cattle, as well as the milk of the latter varies with the lipochrome content of their food. In his splendid paper with Snapper (see van den Bergh and Snapper [1913]) on the lipochrome of the blood serum of man, horses and cattle, he shows unmistakably, however, that he regarded the pigment as originating in the body, although he does raise the question as to the origin of the high pigmentation which he observed in the case of diabetics.

strated, that there is a universal dependence on the diet for the lipochrome carotinoids of animal tissues. One cannot, in fact, account in any other way for the brilliant carotinoid colorations among certain birds, fish and other lower vertebrates.

It must not be forgotten, however, that not all animal lipochromes in the broad sense have been definitely identified as carotinoids. There is at least one (possibly more) red lipochrome widely distributed among animals, as shown in Chapter IV, which does not seem to have an analogue among the plant carotinoids, although it seems to be closely related to them. Such a pigment occurs in the feathers of certain birds, in the salmon muscle, in the hypoderm of Crustacea and elsewhere. Is it a plant carotinoid which has not yet been identified, or is it a modified plant carotinoid? Either of these possibilities is more rational than the possibility that it is actually synthesized by the animals in which it occurs. The red pigment on the legs of the pigeons is probably such a pigment. It is absent from the legs of the young pigeons. In the color of its solutions this pigment strongly resembles lycopin. The writer observed recently that the pigment is strongly epiphasic between petroleum ether and 90 per cent methyl alcohol, but its solutions show no clear absorption spectra. These tests indicate a modified carotin, and yet there is little if any carotin in the blood serum of the pigeon, the great bulk of the pigment being xanthophyll as in the case of the fowl. The problem of modified carotinoids in animal tissues is therefore an important phase of the general hypothesis which must not be overlooked.

There is still another phase of animal pigmentation from the carotinoid standpoint which deserves consideration, namely, that carotinoid pigmentation is not universal, even among herbivorous animals. The writer (1916) first called attention to some variations of this kind among mammals, showing that practically no carotinoids occur in sheep and goats and none in swine. Palmer and Kennedy (1921) showed that there are none of these pigments in the albino rat. Rodents in general, however, do not lack carotinoids, for one finds small amounts in the guinea pig, as shown by van den Bergh, Muller and Broekmeyer (1920). The rabbit is practically, if not entirely devoid of carotinoids, although entirely herbivorous. The same seems to be true of the dog, which is carnivorous, at least by preference. Cats, however, contain traces, as shown by van den Bergh and associates (1920). The general observation that some animals lack lipochromes is not new, for Miss Newbigin used it as an argument against the

hypothesis that the lipochromes are derived pigments, as shown in the quotation given above from her (1898) paper on salmon pigments. The fact is none the less puzzling, however, and offers a very attractive problem for research. There is evidently a physiological factor involved which is characteristic of the species and thus transmitted. Is it an enzyme, possibly an oxidase, in the digestive tract, blood stream or a vital organ, as Gerould (1921) believes to be the case for the carotinoid-free mutant which he has discovered from a normally carotinoid-containing caterpillar? If the carotinoid-free species of animals possess a more highly developed means of oxidizing the carotinoids introduced in their food it should be possible to determine this fact. If the site of this destruction is in the digestive tract the faces of these animals should be devoid of the pigments when the animals are on carotinoid-rich diets. These ideas merely give a hint of the modes of attacking this problem which suggest themselves to the physiological chemist.

An even more fascinating problem is offered by the fact that the cow and the horse resorb the carotin of their rations to the relative exclusion of the xanthophylls although the latter are the more abundant in their food, whereas the fowl resorbs xanthophylls to the relative exclusion of carotin. The failure of cows to respond to the feeding of xanthophyll and the inability of the hen to transmit appreciable amounts of carotin into the egg yolk shows that these results are not to be explained on the grounds that these two species of animals have the power to convert one carotinoid into the other. Palmer and Eckles (1914d) published the results of an attempt to determine whether there is a greater destruction of xanthophyll than carotin along the digestive tract of the cow and whether there is any difference between the action of the natural and artificial digestive fluids on these two classes of carotinoids. In general, the results throw very little light on the fate of the carotinoids during digestion although carotin appeared to show a greater stability; the most significant result secured was that bile dissolves amorphous xanthophyll deposits very readily, while carotin residues are taken up very slowly. This may indicate that the xanthophylls are transported to the liver and there become oxidized while carotin, which forms a complex with a blood protein, escapes this fate. Confirmation of the low solubility of carotin in bile is seen in the finding of Fischer and Röse (1913) that the gall stones of cows contain crystallizable carotin.

No similar studies have ever been undertaken with fowls. The

determination of the relative solubility of their bile on carotin and xanthophylls might lead to suggestive results. No fact has as yet come to light, however, which offers any reasonable basis upon which one can construct an explanation of the rather astonishing divergence between this species and the mammals with respect to the class of carotinoids which predominate in the chromolipoids which color the body tissues.

Summary

The demonstration of the possibility of a general biological relationship between animal chromolipoids and plant carotinoids is a recent achievement. Such a relationship was suggested by earlier workers in isolated cases, but even the chemical identification of certain of the animal lipochromes with plant carotinoids did not suggest to Willstätter and his pupil Escher their possible origin from plant pigments.

Of the earlier investigators Krukenberg and Zopf saw no evidence of such a biological relationship. Miss Newbigin concluded that such a relation existed in specific cases but was not general. Poulton, however, decided that for caterpillars the yellow pigment is derived from "xanthophyll" and the green from chlorophyll.

In addition to Poulton's work, the earlier experiments demonstrating that plant carotinoids can be transferred to animal tissues included Sauermann's (1889) coloring of the feathers of canary birds and fowls, and the egg yolks of the latter, with red pepper pigment. The experiments should be repeated, however, because the results present the apparent anomaly that lycopin (which is apparently the red pepper pigment), the isomer of carotin, is assimilated by the fowl while carotin is absorbed only in traces under the most favorable conditions.

Since these earlier studies Palmer (1914, 1915, 1919) has demonstrated that the carotin of the butter fat, adipose tissue, blood serum, skin secretions, etc., of cattle is biologically derived from the food; that a similar relationship exists between the xanthophyll of egg yolk and fowl tissues and plant xanthophyll; that while there is not an absolute exclusion of xanthophyll by the cow or carotin by the hen, the occurrence of a predominating type of carotinoid in each of the two species is not due to the power of the animals to convert one type of pigment into the other; and that the pigments of human milk fat (and presumably of human tissues in general) may contain either carotin or xanthophylls or both.

These results have been confirmed by subsequent investigations by others and support the thesis that all animal chromolipoids are derived from the carotinoids of the food, and, either unchanged or slightly modified, are the cause of the yellow to red chromolipoid colors of all species of animals.

Carotinoid pigmentation of animal tissues is not, however, universal, even among animals whose diet is normally rich in these pigments, as shown by Palmer (1916). This fact offers a very attractive problem for research. Several methods are suggested for attacking it.

Chapter VIII

Methods of Isolation of Carotinoids

The isolation of the various carotinoid pigments is attended with certain difficulties, which are chiefly mechanical, even if one desires to secure only a few grams of pure crystals. The pigments are all quite intense[1] so that one is readily deceived by the color as to the actual amount of pigment which is present. This fact makes it necessary to carry out the operations involved on a rather generous scale, in order that the yields may justify the effort. The difficulties from a chemical point of view are due primarily to the great ease of oxidation of the pigments and secondarily to the presence of colorless lipoid impurities which unavoidably contaminate the crude products because of the necessity of using the lipoid solvents for the extraction process. The great ease of oxidation of the carotinoids requires the employment of vacuum in carrying out all concentrations and the use of inert gases, if possible, during crystallization processes. The removal of lipoid impurities naturally depends somewhat on the nature of the contaminating substances. Where relatively large amounts of glycerides are involved it is necessary to resort to saponification and subsequent extraction of the unsaponified pigment. As far as carotin is concerned, or its isomer lycopin, this can be done without injury to the pigment. There may be some question whether or not certain of the xanthophylls are altered slightly by this process. For the carotinoid fucoxanthin, however, saponification should certainly be avoided as it is known to form a compound with alkalis under certain conditions. The sterols are removed by washing the crystals with cold solvents, depending upon the carotinoid involved. For carotin, cold alcohol (absolute or 98 per cent) is best, and for xanthophyll cold petroleum ether (b. p. 40-60° C.). Recrystallization must of necessity be resorted to for the final purifications. The details of the operations are mentioned below.

[1] According to Arnaud (1887) carotin is still visible in carbon disulfide in 1 part per million of solvent.

Isolation of Carotin

Carrots. The carrot root naturally suggests itself as the most available source of the pigment carotin. Several methods have been proposed by various investigators, each of which may be indicated briefly.

The method of Arnaud (1886) was to submit the fresh grated carrots to heavy pressure and add an excess of neutral lead acetate to the juice. The precipitate was filtered off, dried in vacuum and added to the pressed carrot pulp, which had also been dried. The combined material was then washed with carbon disulfide at a low temperature. Crude carotin crystallized out of this extract on concentrating it to a low volume and allowing it to stand, if sufficient material had been used. Arnaud obtained three grams from 100 kgs. of carrots by this method. He states that most of the impurities could be washed away from the crude crystalline material by cold petroleum ether.[2] Final purification was secured in Arnaud's work by dissolving the crystals in the least possible amount of carbon disulfide and then adding a large excess of absolute alcohol, in which carotin is practically insoluble. This was followed by a spontaneous crystallization from cold petroleum ether, a final washing with cold absolute alcohol, and drying in vacuum.

Kohl's (1902e) method for isolating carotin from carrots offers certain advantages over that of Arnaud, particularly because smaller quantities of extraction solvent are required. The carrots are sliced and then boiled and pressed. The writer has noticed that there is practically no loss of pigment in either the water in which the carrots are boiled or in the press juice, inasmuch as the heat coagulation of the proteins seems to fix the carotin in the tissues. Kohl washed the first press cake with cold alcohol, pressed it again, ground it and allowed it to dry in the air. A bright orange-red powder resulted if well colored carrots were chosen. Kohl extracted this powder with ether in an extractor of the continuous type until all the pigment was extracted. The ether was removed by evaporation, and saponification carried out in the extraction flask by boiling for an hour with alcoholic potash. The evaporation of the alcohol was carried out in the same flask in a current of CO_2, and the dried soap extracted with chloro-

[2] It is almost necessary to use a fat solvent in this case because of the high content of oil in the carrot root. It is to be expected, also, that some pigment will be lost in carrying out the operation.

form. The carotin was precipitated from the chloroform by an excess of absolute alcohol and purified by recrystallization from petroleum ether. Kohl found that a very good yield of crystals could be secured by omitting the saponification process and merely allowing the concentrated ether extract to evaporate spontaneously. The crystals secured in this manner could be purified further by washing with cold ether followed by cold absolute alcohol.

On forming a concentrated chloroform solution of this residue and adding three volumes of absolute alcohol, the impurities which precipitated immediately could be removed by a quick filtration. On allowing the solution to stand for about 24 hours, pure carotin crystallized out. Kohl does not tell what yields he secured by this method, but he assures us that the product was of a high degree of purity.

A method somewhat similar to that of Kohl was followed by Euler and Nordenson (1908). Fresh carrots in 25 kg. lots were boiled in water for several hours and then pressed. The press cake was ground with sand and dried in thin layers at 50° C., which took about a day. The dried residue was extracted twice with carbon disulfide at 20° C., presumably by agitating with the solvent. The volume of solvent used was not stated. The carbon disulfide was pressed out of the dried carrot pulp and the solvent distilled off of the filtrate, at the last with the addition of much ether. The carrot pulp was now treated with 8 liters of alcohol for several hours, which became deep red with extracted pigment. By diluting with much water and shaking with ether the pigment was transferred to the latter solvent. The two ether solutions of pigment were treated alike. They were first evaporated to dryness and then taken up in a little petroleum ether and three volumes of alcohol added. The precipitated phosphatides were filtered off and the filtrates evaporated to dryness. The combined yields of crude pigment amounted to 26 grams.

The most satisfactory yields of pure pigment were reported by Escher (1909) who obtained ·125 grams from 472 kgs. of dried (5,000 kgs. fresh) carrots. The complete details of the method used have not been accessible to the writer. In general, however, Escher dried the carrots without previous cooking, using a low heat. The dried pulp was ground to a powder and the pigment completely extracted by petroleum ether in a continuous extractor. This extract was concentrated to a low volume under diminished pressure at 40° C. On standing, the carotin crystallized out together with a large amount of colorless impurities. Purification was carried out by fractional pre-

cipitation from carbon disulfide solution with absolute alcohol. In this process the colorless impurities precipitate first and then the pure carotin. By repeating the fractional precipitation pure carotin was finally obtained.

Green leaves. Arnaud (1885) was one of the first to show that crystals of carotin may be secured by a gentle and rapid petroleum ether extraction of vacuum dried, powdered leaves, e.g., spinach, followed by spontaneous evaporation of the concentrated extract. Arnaud found that the waxy substances could be washed away with a little cold ether and the pigment recrystallized from petroleum ether. The interesting feature of this method is the fact that quick extraction of the perfectly dry powdered leaves removes practically no green pigment, and also no appreciable amount of xanthophylls.

Willstätter and Mieg (1907) applied the method of Arnaud to the leaves of the stinging nettle, *Urtica dioica,* in order to isolate carotin on a large scale. The nettle leaves are not so good a source of pigment, however, as spinach, according to these investigators, but their low yield of carotin may have been due to the fact that the leaves were harvested in July when their carotin content, according to Arnaud (1889), is quite low. The details of the operation should be useful for the isolation of carotin from any green, leafy material containing a relatively large quantity of the pigment. One hundred kgs. of powdered nettle leaves (moisture 7.7 per cent) were allowed to stand in contact with 120 liters of cold petroleum ether (b. p. 40-70° C.) in glass flasks for two days. The petroleum ether was filtered off on a Büchner funnel and the residue on the filter washed with 60 liters of petroleum ether. It is stated that no xanthophyll was present in the greenish yellow extract. The small amount of chlorophyll present was removed first. This was done by shaking the extract gently with a little concentrated alcoholic potash, being careful to avoid an emulsion. The alkali was removed by washing with water, but here again care had to be taken to shake the mixtures very gently because the petroleum ether solution still contained considerable fat-like material. In the writer's experience these processes of removing the chlorophyll and washing out the alkali are likely to be somewhat tedious. When they are completed one can proceed to the evaporation of the extracts, which must be carried out in vacuum. In Willstätter and Mieg's experiments the 200 liters of petroleum ether were evaporated to about three liters before setting aside for the carotin to crystallize out. It

is stated that when the petroleum ether had evaporated the carotin was found as glistening crystals embedded in a dark, waxy mass.

The removal of the waxy impurities and the final purification are carried out as follows. The mixture of wax and pigment is carefully shaken with three liters of the lowest boiling petroleum ether (presumably that boiling under 50° C.) which removes the bulk of the wax. The solution is filtered, leaving the carotin on the filter but still somewhat contaminated with phytosterol and other colorless substances. According to Willstätter and Mieg the carotin lost in the filtrate can be recovered through precipitation by alcohol, but the details of this recovery are not given. The final purification is carried out as in the case of the isolation of carotin from carrots, namely, by dissolving in a small amount of carbon disulfide, in which a part of the colorless substances do not readily dissolve, and then by adding absolute alcohol cautiously to secure the fractional precipitation of the other impurities from the carotin. The colorless substances come down first and can be quickly filtered off. Then the carotin precipitates as sparkling crystals. At this point the yield was a little over 3 grams of crystals in Willstätter and Mieg's work. This is mentioned because it gives a good idea of the scale on which it is apparently necessary to operate in order to secure even small quantities of relatively pure pigment. With the facilities available in most laboratories 10 kgs. of dried, highly pigmented leaves would be somewhat burdensome to carry through rapidly enough to avoid loss of pigment by oxidation. The yield of relatively pure pigment by this method would not be over 0.5 grams at the most, using highly pigmented spinach leaves as the source of material.

The final purification of carotin is carried out in the usual manner, namely, by repeated precipitations from carbon disulfide by absolute alcohol and a final crystallization from the lowest boiling petroleum ether. The final yield of perfectly pure pigment would naturally be somewhat less than the figures mentioned above.

The most tedious features of this process are the removal of the small amount of extracted chlorophyll from the dried leaves, and the final purification of the crude carotin. It is not likely that the fractional precipitations and recrystallizations can well be avoided. It seems feasible, however, to substitute a more direct method for removing the chlorophyll. Tswett (1906b) has shown that if a petroleum ether solution of chlorophyll and carotin is shaken with an excess of dry, finely divided $CaCO_3$, inulin or sucrose, the chlorophyll is com-

pletely adsorbed, leaving the carotin in solution. Any carotin held mechanically by the adsorbing material can be washed out with petroleum ether without removing the adsorbed chlorophyll. It would seem entirely practicable to apply these facts to the isolation of crystalline carotin from leaves. The removal of the chlorophyll could be postponed until the bulk of the petroleum ether was distilled off and before the final concentration and crystallization of the pigment. According to the laws of adsorption it may be expected that the adsorbing material will also remove a certain amount of some of the other impurities. In applying this method care must be taken to choose only the most finely divided adsorbing agent.

The problem of securing pigment solutions from fresh or dried plant tissues merely for macroscopic examination is much less complicated. Fresh tissues should first be macerated. Tswett recommends the use of a little $CaCO_3$ or MgO in connection with the maceration to neutralize the acids in the plant sap. In order to choose the proper solvent it is well to have in mind certain rules laid down by Tswett (1906b) for the action of the various solvents upon the carotinoid and chlorophyll pigments in plants. According to Tswett the solvents commonly used are divided into three groups according to their relations toward the leaf pigments.

1. Alcohol (methyl, ethyl, amyl), acetone, acetaldehyde, ether, chloroform.—These solvents acting on fresh (macerated) or dried leaves dissolve out all the pigments equally and completely.

2. Petroleum ether and petroleum benzine (low or high b. p. petroleum ether).—Fresh leaves (macerated) give more or less yellow extracts when treated with these solvents. The chief pigment is carotin but traces of other pigments are also extracted. Leaves dried at low temperature likewise give up their carotin to these solvents, and in somewhat purer condition. Plant tissues which have been cooked, or only warmed to a moderately high temperature, however, give green extracts when macerated with these solvents.

3. Benzene, xylene, toluene and carbon disulfide.—These solvents act intermediately between the first and second groups. For the extraction of all the chlorophyll and carotinoid pigments Tswett recommends petroleum ether containing 10 per cent absolute alcohol for fresh leaves and petroleum ether containing 1 per cent alcohol for dry leaves.

Animal fat. It is manifestly impossible to secure carotin in appreciable quantities from animal fat, like butter fat, or from the highly

pigmented adipose tissue which one finds in certain breeds of dairy cattle. At the most butter fat contains little more than 0.005 per cent carotin, and in many cases considerably less than this amount. Ten to 20 kgs. of fat would therefore be required to secure 0.5 grams of pigment, assuming that all of it could be recovered. The problem is rendered still more difficult by the fact that the fat must be completely saponified before the pigment can be extracted; and, if it is necessary to use a procedure in connection with the saponification and extraction of pigment from large quantities of fat such as has been found practicable for small quantities, the volumes of soap solution and ether required for the operation would soon reach a magnitude all out of proportion to the facilities of the best appointed laboratories. To be specific, at least 120 liters of ether would be necessary to secure the carotin from 10,000 grams of fat, and inasmuch as the yield could not be over a few tenths of a gram of crystalline product the mechanical difficulties involved would not justify the attempt.

It is readily possible, however, to obtain sufficient carotin from animal fat for a macroscopic study of the chemical and physical properties of the pigment. Twenty to thirty grams of well colored butter fat or rendered adipose tissue fat are ample for such a study. The butter fat must not be artificially colored, the pure rendered butter fat from Jersey or Guernsey cows on a fresh pasture-grass diet being best suited for the experiment. The fat must first be saponified; and, in this connection, an important precaution must be taken, namely, to avoid the use of alcohol which has not been completely purified from aldehydes which produce yellow to red colored resins with alkali.[3] The resins thus formed follow the carotinoids in their isolation and interfere greatly with the study of the properties of the pigments.

For the saponification of the fat, 2 cc. of colorless 20 per cent alcoholic potash is added for each gram of fat and the mixture allowed to boil for about one hour under a reflux condenser. The

[3] Ethyl alcohol is especially likely to contain such impurities. It can be purified best by treatment with silver nitrate, in which about 2.0 grams of crystals are added to 4 liters of alcohol and allowed to stand, with shaking, for several days. 200 g. unslaked lime are now added to precipitate the AgO, neutralize the acids and remove any excess water. The lime can now be filtered off and the filtrate distilled. Usually one such treatment will prepare an excellent 98 per cent alcohol which will show no coloration on boiling in the presence of 20 per cent KOH. Should a color develop under these conditions the purification must be repeated.

Methyl alcohol can also be used for the saponification of the fat, but it, also, must show no coloration when a 20 per cent KOH solution of the alcohol is boiled.

resulting soap is dissolved in three volumes of distilled water. After cooling, this solution is shaken with an equal volume of pure ether in a separatory funnel. The extraction is repeated with a fresh volume of ether equal to one-half the volume of soap solution. The soap should now be colorless.[4] The combined ether extracts are now washed many times with an excess of water, carefully at first to avoid emulsions, and more vigorously with subsequent washings. When the wash water no longer reacts alkaline to phenolphthalein, the ether solution is dried by shaking with neutral, fused $CaCl_2$ or anhydrous Na_2SO_4 for a few hours, decanted or filtered from the inorganic drying agents and evaporated to dryness in a dry vacuum. Little or no heat need be applied because of the rapid volatilization of ether under diminished pressure. The residue consists of pigment mixed with large quantities of cholesterol and traces of other unsaponifiable matter. According to Steenbock (1921a) and others, the fat-soluble vitamine in butter fat is present in this fraction. The cholesterol can be removed by the digitonin method of Windaus (1909), by dissolving the residue in warm 95 per cent alcohol and adding an excess of a hot one per cent solution of digitonin in 90 per cent alcohol. This procedure is not necessary, however, for the study of the chemical and physical properties of the pigment.

The examination of pigment isolated from animal fat in the above manner must be made at once unless facilities are available for keeping the pigment in an atmosphere of inert gas. Kohl (1902b) states that crystalline carotin can be protected completely from oxidation, even in the sunlight, if placed under glycerin. The writer has never tried this method for crude preparations of pigment from animal tissues, so is unable to vouch for its usefulness for pigments prepared by the method just given.

Blood serum. The blood serum of man and certain animals may be relatively rich in carotin, giving it a golden yellow color. While this material can not be expected to serve as a suitable source of pigment in large quantities the pigment can be isolated in sufficient amounts for chemical examination without great difficulty. Serum or plasma free from erythrocytes must first be obtained. This may be done either by allowing the blood to clot and permitting the serum

[4] Many of the early workers who saponified their plant or animal extracts evaporated the alcohol and extracted the dried soaps with the solvents, or carried out the extractions with soaps which had been salted out of aqueous solution with NaCl. In the writer's experience these procedures are not advantageous when working with pure animal fats which contain carotinoids.

to separate when the clot contracts, or by defibrinating the freshly drawn blood by whipping it vigorously, filtering off the fibrin and centrifuging the erythrocytes from the defibrinated plasma, or merely by drawing the blood into sufficient saturated potassium oxalate or sodium citrate solution to prevent clotting and throwing down the erythrocytes from the oxalated or citrated blood with the centrifuge. Each of the three preparations, namely, serum, defibrinated plasma or oxalated (or citrated) plasma serve equally well for the isolation of the serum carotinoids.

In most cases carotin, when present in blood, appears to be in some sort of physico-chemical combination with a fraction of the albumin in colloidal solution in the blood. Whatever the explanation of the state of the pigment in the blood may be in these cases, the fact remains that when this occurs the direct extraction of the pigment with ether, petroleum ether, chloroform, carbon disulfide or any of the usual carotin solvents is impossible. However, if the serum is first treated with an equal volume of alcohol, the carotin can be readily extracted by shaking with the solvents mentioned. Based on this fact the writer devised the following method for extracting the carotin from blood serum: Clear serum or plasma is mixed with an excess of plaster of Paris, using about 40 grams of the $CaSO_4$ for each 10 cc. of serum. The damp powder is transferred to a flask, alcohol added equal to the volume of serum and thoroughly mixed with the plaster of Paris mass. An equal volume of low boiling petroleum ether is now added and vigorously shaken with the mass. On standing, the petroleum ether rises to the surface, giving an almost quantitative extraction of the carotin. The extract can be readily poured off and the extraction repeated with fresh petroleum ether in order to insure a complete extraction.

Reference has already been made to the manner in which blood carries the carotin. Until recently the writer held the view that carotin is always present in some sort of combination with an albumin fraction in the serum. So far as his experience with the blood of cattle and horses is concerned this view still holds. However, he has recently examined the blood of several diabetics on vegetarian diets containing much green food in which this carotin-albumin combination did not appear to exist. At least the pigment, which proved to be carotin, or at any rate to have the relative solubility and other chemical properties of carotin, and not xanthophyll, was readily and completely extracted from the serum merely by vigorous shaking with

fresh portions of pure ether. Even in these cases extraction of the pigment could be facilitated by diluting the serum with two volumes of water and adding an equal volume of methyl alcohol before shaking with ether.

In the writer's experience with cattle and horse serum the evidence for a carotin-albumin combination of some kind rests upon a number of easily demonstrated facts, among which are the following. The fat solvents will extract little if any pigment from serum even after great dilution with water. When the globulins and albumins in the serum are fractionally precipitated by increasing concentrations of ammonium sulfate, the carotin follows the albumin fractions. In fact it is possible to roughly isolate an albumin which carries the carotin in firm combination, which, like the serum itself, will not give up its pigment to the fat solvents until first treated with alcohol, indeed unless alcohol is present. The lead, silver and mercury salts of the protein act in the same manner. After coagulation with alcohol and drying it was found, in one test at least, that alcohol had to be added before petroleum ether would extract the pigment from the protein. This albumin, moreover, seems to have a more or less definite heat-coagulation point of 86° C., when in half-saturated ammonium sulfate solution. This property can therefore be used for the isolation of the pigment-carrying protein.

The isolation of the carotin-albumin complex can be carried out as follows:—The serum is first freed from globulins by adding an equal volume of saturated ammonium sulfate solution. These are filtered off on a Büchner funnel, using suction, and thoroughly washed with half saturated ammonium sulfate solution. The combined filtrate and washings are then carefully heated to a temperature of 79° C., at which temperature the bulk of the albumins are coagulated. Some carotin is lost in this coagulum, but with serum rich in carotin the filtrate from these proteins will have a golden yellow color. The carotin-albumin fraction is secured from this filtrate either by salting it out by any of the albumin precipitants (complete saturation with ammonium sulfate is best) or by heating to 86° C. In either case the precipitate will have a deep yellow color and the amount obtained will be very small in comparison with the proteins which have been precipitated as globulins and albumins in the preliminary operations. The protein can be redissolved after salting out, and the aqueous solution thus obtained exhibits all the properties of blood serum so far as its relations to fat solvents and the extraction of

the pigment are concerned. The writer has long been impressed with the possibility of throwing light upon the formation of milk fat through a study of this interesting complex in the blood of cattle, inasmuch as it is unquestionably the source from which the milk fat derives its natural pigment. The failure of the fat solvents to remove the pigment from this protein complex by direct extraction indicates that the pigment becomes a part of the milk fat through a process much more deep-seated than a mere solvent action. It seems very probable, therefore, that this caroto-albumin plays some important part in the process of fat synthesis in the mammary gland of the cow.

Isolation of Xanthophylls

Green leaves. It was clearly shown in Chapter II that a group of xanthophyll pigments accompany chlorophyll and carotin in green leaves. It is not known, however, whether the crystalline xanthophyll which can be isolated from the green leaves of plants is a mixture of the xanthophyll isomers or consists of the major xanthophyll constituent, the xanthophyll *a* of Tswett. The evidence on both sides of the question was presented in Chapter II.

Willstätter and Mieg (1907) were the first to isolate crystalline xanthophyll in quantity. Their method was as follows. Air-dried, powdered nettle leaves were extracted with cold 95 per cent alcohol, which extracted the chlorophylls and xanthophylls, but very little of the carotin. The extract was treated in the cold with KOH, converting the chlorophylls into chlorophyllins, which precipitated in part directly from the alcoholic solution and partly on addition of much ether. The alkaline alcoholic-ether solution was shaken with successive portions of fresh water until all the green color was removed from the ether layer. The combined ether solutions obtained in this way from 100 kgs. of dried nettle leaves were concentrated to a volume of 6 liters and after more washing with alcoholic potash and water and drying with anhydrous sodium sulfate, were mixed with two volumes of petroleum ether. This precipitated the xanthophyll and a considerable amount of colorless, high molecular weight alcohol, the xanthophyll coming down as a reddish-yellow precipitate.

To remove the impurities from the precipitated xanthophyll Willstätter and Mieg boiled the precipitate with 1200 cc. of acetone, which left a part of the colorless substance undissolved. The warm acetone solution was treated with about two volumes of methyl alcohol. In

the course of two days the xanthophyll crystallized out at room temperature as yellow to orange-red tablets with a brilliant steel-blue reflection. Willstätter and Mieg secured a yield of 12 grams of crude crystals from the 100 kgs. of dried nettle leaves. Further purification was obtained by crystallization from boiling methyl alcohol, from which the crystals come down with one molecule of alcohol of crystallization, or by dissolving in the least possible amount of chloroform and adding an excess of petroleum ether, in which the xanthophyll is almost insoluble.

Jörgensen and Stiles (1917) have described what appears to be a more convenient method for isolating crystalline xanthophyll, which also gives higher yields than the method of Willstätter and Mieg. They prefer the dry, powdered nettle leaves because of their excellent keeping quality, but in this respect spinach, which is richer in carotinoids, should serve equally well as a source of pigment.

In Jörgensen and Stiles' method the air dried, powdered leaves are submitted to a final drying in vacuum, over H_2SO_4. About 500 grams of this powder are placed on a filter paper in a Büchner funnel 24 cm. in diameter and sucked to the paper with a strong water pump or vacuum pump. To get the best results the powder must be thoroughly dry and be sucked in a coherent mass not more than 5 cm. deep on the funnel. Half a liter of 80 per cent acetone is now allowed to permeate the powder on the filter for 5 minutes without the use of the pump. Then 250 cc. of solvent are added and slowly sucked through with the pump. After 5 minutes another 250 cc. portion of solvent is added and sucked through with the pump for 10 minutes. This operation is repeated with two further 250 cc. portions of 80 per cent acetone, and finally the pump is allowed to act as strongly as possible until the powder is sucked dry. The 1,500 cc. of solvent used give 800 to 900 cc. of extract.

When the extract has been obtained from 2 kgs. of dry powder in this manner, the fractions are combined and washed free from many impurities and finally from acetone by the following procedure. The acetone solution is added in two successive portions to 4 liters of petroleum ether (sp. g. 0.64 to 0.66) in a separatory funnel of 7 liter capacity. Water (0.5 liters) is added with each of these additions while the funnel is being gently rotated, and after separation into two layers the lower layer is drawn off and discarded before the addition of the second portion. The petroleum ether layer is now mixed with two successive liters of 80 per cent acetone solution, the

acetone being removed each time by adding 4 successive liters of water with gentle rotation of the liquid and drawing off the lower layer each time.

The petroleum ether solution now remaining contains the xanthophylls, the chlorophylls and the carotin. The xanthophylls, with some chlorophyll, are removed by shaking with three successive additions of 2 liters of 80 per cent methyl alcohol. After each addition and shaking the methyl alcohol layer is removed. If the last extract is still considerably yellow additional extractions are made until the alcohol layer is practically colorless. The xanthophyll in the combined methyl alcohol extracts is next freed from chlorophyll by transferring to ether in the following manner: 4 to 5 liters of ether, a quantity of water and 30-50 cc. of concentrated methyl alcoholic potash are added, and the mixture shaken. The liquids are allowed to separate, the lower layer is drawn off and discarded and the ether washed with water until no more green color is extracted. The ether is now dried with anhydrous Na_2SO_4, evaporated in vacuum to a volume of 30 cc., 200 to 300 cc. of methyl alcohol added, and the ether removed completely by further concentration in vacuum. Xanthophyll precipitates out on cooling the hot, concentrated methyl alcohol, the addition of a little water helping the precipitation. The yield of crude xanthophyll by this method is stated by Jörgensen and Stiles to be 0.8 grams from 2 kgs. of dried nettle leaves which is over three times as much as Willstätter and Mieg secured by their method. The method just described has the additional advantage that the xanthophyll-free petroleum ether can be used for the isolation of carotin.[5]

The foregoing methods are best suited for the isolation of crystalline xanthophyll in quantity. A solution of mixed xanthophylls for macroscopic examination can be secured by the following simple procedure. About 25 grams of dried powdered leaves or fresh leaves

[5] The method recommended is to wash the petroleum ether, now consisting of about 3.5 liters, four times with two liter portions of water to remove the last traces of acetone and methyl alcohol. As the last traces of these solvents are removed the chlorophyll present in the petroleum ether precipitates as a fine suspension. A little anhydrous Na_2SO_4 is added to take up the water and then 150 grams of $CaCO_3$, and the solution finally filtered through a layer of $CaCO_3$ on a Büchner funnel. This treatment takes out the chlorophyll suspension. The filtrate is evaporated in vacuum at 40° C. and the oily residue treated with 300 cc. of 90 per cent alcohol. The carotin begins to crystallize out immediately and is complete on standing in the cold. Purification is effected by shaking up the crystalline mass with 200–300 cc. of petroleum ether and filtering quickly and repeating the washing with a mixture of two parts of petroleum ether and one part of absolute alcohol. The yield of 0.25 grams from two kgs. of dried nettle leaves is much greater than Willstätter and Mieg secured.

which have been macerated with emery in the presence of $CaCO_3$ or MgO (to neutralize plant acids) are allowed to stand in contact with pure carbon disulfide in a stoppered flask for 24-48 hours. The solvent is filtered off and evaporated to dryness in vacuum. The residue is boiled for thirty minutes with 50 cc. of 20 per cent methyl or ethyl alcoholic potash (using only solutions which alone give no coloration whatever on boiling). After cooling, 150 cc. of distilled water are added and the mixture shaken with 200 cc. of pure ether in a separatory funnel. After the two layers have separated the lower greenish layer is drawn off and shaken with 100 cc. of fresh ether. A third extraction with fresh ether should not be necessary, but can be tried to insure the complete extraction of the carotinoids. The combined golden yellow ether extracts, which may have a slight green tinge, are washed with successive equal portions of distilled water until the washings no longer react alkaline to phenolphthalein. The ether may now be filtered through a layer of powdered anhydrous Na_2SO_4, to remove the water. The filtrate is evaporated to dryness in vacuum and the residue taken up at once in 100 cc. of hot petroleum ether (b. p. 30-50° C.). After cooling, the solution is shaken with successive 100 cc. portions of 80 per cent methyl alcohol until no more color is extracted. The combined methyl alcohol solutions contain the xanthophylls. On dilution with water to form a 25-30 per cent alcohol solution ether will now extract these pigments. After washing the ether free from alcohol with water and drying with Na_2SO_4, the ether can be evaporated off in vacuum and the pigmented residue used for an examination of any of the usual xanthophyll properties.

Egg yolk. The large amount of protein, fat, lecithin and other lipoids in egg yolk presents certain rather difficult problems in the isolation of the xanthophyll pigment present. The isolation was accomplished, however, by Willstätter and Escher (1912) in the following manner, but not without loss of a great deal of pigment, as can be readily seen. Egg yolk weighing 100 kgs., representing 6,000 eggs, was beaten up and 6 kg. portions placed in stone jars with 7 liters of methyl alcohol to coagulate the protein. The coagulum was separated by means of the centrifuge, the alcohol, it is stated, being almost free from color. Each portion of coagulum, amounting to a little over 5 kgs., was thoroughly mixed with 3 liters of acetone, and the golden yellow extract sucked off through a sand filter. After the coagulum from each 6 kg. portion of egg yolk had been extracted

in this way the residue (94 kgs. in all) was divided into portions of 2.8 kgs. and each portion shaken twice with fresh two liter quantities of acetone in a shaking machine for one hour, the acetone being sucked off each time through a sand filter. Practically all the color was extracted by this means.

All the acetone extracts were now combined and amounted to 200 liters. About 2.25 liters of oil settled out on standing. Although highly colored it was discarded. The next problem was to remove the phosphatides and cholesterol. The phosphatides were removed by mixing each 6 liter portion of acetone extract with 0.5 liter of petroleum ether (sp. g., 0.64-0.66), and adding three volumes of water carefully, to avoid an emulsion. The lower watery acetone layer was drawn off after standing a day and the dark brown, thick oily syrup rinsed out with petroleum ether. Twenty liters, in all, of this oily material were obtained. Large clumps of almost colorless phosphatides, amounting to nearly 2 kgs. were thrown down by adding 2 volumes of acetone to the petroleum ether solution of this syrup. The pigmented acetone-petroleum ether solution was decanted, filtered through linen, and freed from acetone again by washing with water, first by decantation and finally by direct addition of water, allowing about one hour between each addition. The reddish-brown petroleum ether solution was now filtered through fused Na_2SO_4 and the filtrate concentrated to 2 liters at 30-35° C., in vacuum, i. e., until the syrup set to a crystalline mass of cholesterol. This was filtered off and the deep colored filtrate diluted with 4 liters of petroleum ether (b. p. 30°-50° C.). On standing in the ice box for a few days most of the pigment crystallized out as a bright red blanket of very fine needles. The yield of crude pigment amounted to 4 grams. The purification of the pigment was described in Chapter VI. It is of interest that the method of isolation used by Willstätter and Escher shows that a portion, at least, of the egg yolk carotinoids are not present dissolved in fat. It was not found necessary to resort to a saponification of the extracts in order to isolate crystals of pigment.

The separation of sufficient egg yolk pigment for macroscopic examination can be effected in a satisfactory manner from a single well-colored egg yolk. For this purpose the following procedure gives very satisfactory results. The raw yolk is thrown into 100 cc. of acetone, and, after heating to boiling, filtered to remove the coagulated, colorless proteins. The filtrate is evaporated and the residue saponified with 50 cc. of 20 per cent methyl alcoholic potash solution

at boiling temperature for about one hour, taking care to use alcoholic potash which itself gives rise to no color on heating. The pigment is extracted from the saponified material using the procedure given for isolating the pigment of butter fat. The ether solution of pigment is dried by filtering through a layer of anhydrous Na_2SO_4 and evaporated to dryness in vacuum. The chief impurity in the residue will be cholesterol. By dissolving in the least possible amount of hot methyl alcohol and cooling to a low temperature a great deal of the cholesterol will precipitate out and can be removed by filtration. The cholesterol which remains will not interfere with the examination of the pigment. In the writer's experience egg yolk pigment prepared in this way will invariably show the presence of a small amount of pigment which cannot be extracted from petroleum ether by 80 per cent methyl alcohol, indicating that carotin-like pigments are not entirely absent from egg yolk.

Blood serum. It is not necessary to dwell at length on the isolation of xanthophyll from blood serum in view of the detailed description already given of the procedure to be used for isolating carotin from blood. One or two points, however, should be emphasized. Xanthophylls are found most abundantly in the blood serum of fowls, as has already been pointed out. This does not mean, however, that blood rich in carotin, like cattle blood, is necessarily devoid of xanthophylls. In order to show the presence of these pigments in cattle blood it is necessary to extract 200-300 cc. of desiccated (with $CaSO_4$) serum completely with ether as well as with petroleum ether, after treating with alcohol. The combined pigments from well colored serum will show the presence of xanthophylls when submitted to the phase test or analyzed by means of the chromatograph.

It has been the writer's invariable experience with the blood of fowls that the xanthophylls present can be readily extracted by direct shaking of either the fresh or desiccated serum with ether. The experience of van den Bergh and Muller (1920) has been contrary to this, these investigators finding a number of cases in which ether extraction failed. No explanation is as yet apparent for this divergence in our experiences. However, in view of the fact that it appears possible for cases to occur in which ether extraction alone fails to remove the pigment the writer advises that desiccated blood serum, in which xanthophylls are suspected to exist, be extracted first with ether, then treated with alcohol and the ether extraction repeated.

Until recently the writer believed that the direct extraction of caro-

tinoid from blood serum by ether was a criterion of its xanthophyll character. The instances of carotin extraction from human blood serum which the writer has already mentioned show, however, that this is not a safe basis for judging the character of the pigment present.

Isolation of Lycopin

Several investigators have described the isolation of lycopin, which is the characteristic red pigment of tomatoes, red peppers, the pulp of the watermelon and a number of tropical fruits. The method to be described here, however, is that used by Willstätter and Escher (1910), who first showed that this pigment is a true isomer of carotin. These investigators first attempted to use the fresh fruits as the source of pigment, but when they found that 135 kgs. of tomatoes yielded only 2.6 kgs. of dry matter from which only 2.7 grams of crystalline lycopin could be obtained, they decided on a canned preparation of concentrated tomato soup of Italian make as better suited for their work.

Starting with 74 kgs. of the condensed tomato purée, it was first dried in 8 kg. portions by shaking with 4 liters of 96 per cent alcohol, collecting the coagulum and repeating the operation with two or three liters more of the alcohol. The coagulum was now pressed as dry as possible and finally dried completely on the steam bath before grinding to a powder. The total yield of dry powder was 5.6 kgs. This was completely extracted with carbon disulfide in a continuous extractor and the extract evaporated to dryness using diminished pressure as far as possible, and finally at a temperature of 40° C. in a water bath. The residue was now treated with 3 volumes of absolute alcohol, transferred to a suction filter and washed with petroleum ether. The yield of crude pigment amounted to 11 grams. The purification of the pigment was accomplished in much the same manner as carotin is purified.

Isolation of Fucoxanthin

The characteristic algæ pigment, fucoxanthin, whose chemical relation to the carotinoids was discovered by Willstätter and Page (1914) was isolated in quantity by them in the following manner. Fifteen to 20 kgs. of the fresh algæ (*Phaeophyceæ*) were extracted with 40 per cent acetone, using 2 liters for each kg. of algæ. This extract was

discarded. The extracted material was pulverized and extracted at once (i. e., within a few days) with 5 portions of 85 per cent alcohol. The last four fractions were combined (the first being discarded) and amounted to 25 liters for the 20 kgs. of algæ. This solution was next shaken with $CaCO_3$ to neutralize acids, and decanted from the settled chalk. The solution was now diluted with water, using 3.4 volumes for each 10 volumes of extract. The chlorophyll which precipitated was allowed to settle out and the supernatant fluid, amounting to 40 liters in all, used as the mother liquor for the isolation of the fucoxanthin. This was accomplished as follows:

Four-liter portions were treated with one liter of a mixture of ether and petroleum ether (b. p., 30°-50° C.), 3:1, and 1.5 liters of water added. The ether layer which took up the pigment was then washed very carefully with water (to avoid emulsions) in order to free it from the acetone which was used to extract the pigments from the algæ. The petroleum ether was then concentrated to 0.5 liter in vacuum, diluted with an equal volume of ether and shaken with 70 per cent methyl alcohol saturated with petroleum ether. This removed the fucoxanthin, together with some xanthophyll. The xanthophyll was removed by shaking the methyl alcohol with an equal volume of a mixture of petroleum ether and ether (5:1). The fucoxanthin was then transferred to ether, the ether solution was concentrated to a thick syrup and about 1 liter of low boiling petroleum ether added. The precipitate of crude pigment obtained amounted to about 2 grams from each 4 liter portion of mother liquor, representing about half the total pigment present. The crude pigment was purified by recrystallization from methyl alcohol, giving crystals containing three molecules of methyl alcohol of crystallization, which could be removed in vacuum. Solvent-free crystals were obtained by precipitation from ether with low b. p. petroleum ether.

Isolation of Rhodoxanthin

This pigment, as explained in an earlier chapter, appears to be a red xanthophyll. It was discovered by Monteverde (1893) in the Russian pond-weed, *Potamogeton natans,* later by Tswett (1911) as the cause of the winter red color of the arbor vitæ, *Thuja orientalis,* and a little later by Monteverde and Lubimenko (1913b) in the arillus of the seed of the yew, *Taxus baccata.* The isolation of crystals for macroscopic examination can be carried out as follows,

according to Monteverde and Lubimenko. The dried material is first extracted with absolute alcohol, which takes out all the pigments. The extract is next treated with saturated $Ba(OH)_2$ solution, which precipitates all the pigments. The precipitate is extracted with alcohol, which extracts the rhodoxanthin, together with the carotin and xanthophylls, if present. The carotin is removed by shaking with petroleum ether. This removes a little of the rhodoxanthin, but the bulk of the pigment remains in the alcohol. The rhodoxanthin shows great crystallizability, and can be obtained in crystalline form merely by evaporating the alcohol solution, whereas the xanthophyll is left as an amorphous deposit. The rhodoxanthin crystals can be washed free from most impurities by petroleum ether, in which the pigment, like xanthophyll, is practially insoluble.

Summary

The principles involved in the isolation of the several carotinoids from plant and animal tissues are described in this chapter. The methods are also given in detail for the isolation of crystalline carotin in quantity from carrots and green leaves, and of its isolation from animal fat and blood in sufficient quantity for macroscopic study.

The evidence is presented for the existence of a carotin-albumin complex in blood serum, and the method described by which this can be isolated. It is pointed out that this pigment-protein material may play an important part in the process of fat synthesis in the mammary gland of the cow and that its further study may therefore throw light on the formation of milk fat.

Methods are described in detail whereby crystalline xanthophyll can be secured in quantity from green leaves and egg yolk, as well as methods for separating the pigment in small quantity from eggs and blood for macroscopic study.

It is pointed out that xanthophyll, in contrast with carotin, is, in most cases, readily extracted from blood serum by vigorous direct shaking with ether. This is not a safe basis, however, for judging the character of the carotinoid present in blood.

The isolation of crystalline lycopin in quantity from tomatoes is described, as well as the isolation of fucoxanthin from brown seaweeds. The method is given for securing crystals of rhodoxanthin.

Chapter IX

General Properties and Methods of Identification of Carotinoids

The preceding chapter shows clearly the difficulty of securing appreciable quantities of the carotinoids in crystalline form. It is not difficult, however, to obtain solutions of the carotinoids which show a number of characteristic properties that can serve for the identification of the pigments. For the sake of convenience, therefore, the properties of the carotinoid solutions and the properties of the crystals of the pigments will be presented separately. It may be stated that the facts to be presented have been drawn largely from the observations of Kohl, Tswett, Willstätter and his coworkers, together with the writer's own experience with these pigments. These researches have already been referred to specifically a number of times in the preceding pages.

Properties of Carotinoid Solutions

Carotin. Carotin forms well colored solutions in ether, chloroform, petroleum ether, benzene, carbon tetrachloride and carbon disulfide, as well as in ethereal and fatty oils and oleic acid. The carbon disulfide solutions are characterized by their red orange to blood red color. The solutions in the other solvents mentioned are yellow to golden yellow, depending on the concentration. Amorphous carotin or carotin in the presence of lipoids, will dissolve in 95 per cent alcohol or even absolute alcohol, giving yellow to golden colored solutions, especially if hot alcohol is used for dissolving the pigment. Very faintly colored solutions are secured with dilute alcohol, as a rule. Carotin crystals are insoluble in absolute alcohol, but oxidation of carotin as well as melting the crystals greatly increases the solubility in this solvent. At the same time the solubility in carbon disulfide decreases. According to van den Bergh, Muller and Broekmeyer (1920) colloidal, aqueous solutions of carotin can be obtained by a slow evaporation of a concentrated alcoholic solution to which several volumes

of water have been added. This evaporation must be carried out in vacuum aided by a little heat.

Solutions of carotin are unaffected by boiling with alkalis, and may be recovered unchanged from such solutions. When dissolved in petroleum ether and carbon disulfide, carotin is not adsorbed by finely divided substances like calcium carbonate, inulin or powdered sucrose. However, according to Tswett (1906b), carotin is adsorbed from petroleum ether solution by finely divided $HgCl_2$, $CaCl_2$ and PbS. Miss Stephenson (1920) has reported that butter fat dissolved in three volumes of petroleum ether can be completely decolorized of its carotin by shaking for several hours with a special birchwood charcoal, using 2.5 grams per 100 grams of fat. The writer has experimented with a number of decolorizing carbons without being able to duplicate this result. In strictly adsorption experiments in which there was no indication that decolorization was due in part to oxidation of the carotin, it was found that at least five times this amount of the most effective carbon so far obtainable was required to completely adsorb the carotin. The fact that carotin is not adsorbed from its petroleum ether solution by calcium carbonate distinguishes the pigment sharply from some of the other carotinoids, particularly the xanthophylls. As a corollary to this property, when a petroleum ether or carbon disulfide solution of carotin is filtered through a column of tightly packed, perfectly dry calcium carbonate, which has first been moistened with the solvent (Tswett's chromatographic analysis) the carotin passes through unadsorbed. When carbon disulfide is used the zone of carotin usually has a characteristic rose color.

Alcoholic solutions of carotin are not characterized by giving color reactions on addition of concentrated HCl, HNO_3 or H_2SO_4 as are certain of the xanthophylls, although in most cases the golden yellow solutions change slowly to a deep green before fading. The complete fading of this green solution may require several days. The addition of NH_4OH to the green solution will restore the yellow color, although the color is somewhat lighter than the original, and the green color can be renewed by adding acid. Solutions of carotin in oil or melted fat give a beautiful green color reaction on dissolving a very small crystal of Fe_2Cl_6 in the warm oil or fat. A few tenths of a milligram of the iron salt is sufficient to add to 5 cc. of well colored oil. The reaction is very delicate, and is given by xanthophylls as well as carotin. Palmer and Thrun (1916) found that this reaction is caused by the oxidation of the carotinoid, the

iron salt being at the same time reduced to the green $FeCl_2$. Husemann (1861) apparently discovered this reaction when adding Fe_2Cl_6 to an alcoholic solution of carotin, but it is doubtful whether the reaction is applicable to alcoholic solutions of the pigment because of the fact that alcohol itself will reduce the red ferric salt to the green ferrous compound.

Gill (1917) has found that the so-called Crampton-Simons test for palm oil, in which a bluish-green color reaction is given by an acetic anhydride reagent, is due to carotinoids in the oil. Gill's idea that carotin alone is involved is hardly justified, because the color reactions of carotin are in general shared by the other carotinoids.

Solutions of carotin in alcohol which has been diluted with water to a concentration of 80 to 90 per cent alcohol are characterized by giving up the pigment quantitatively to carbon disulfide and petroleum ether. Conversely, carotin in petroleum ether is unaffected by shaking with 80 to 90 per cent alcohol, even 92 per cent methyl alcohol failing to extract any pigment from the petroleum ether solution. These properties of carotin, especially the relatively great solubility in petroleum ether in comparison with diluted alcohol, serves to distinguish carotin sharply from the xanthophylls, rhodoxanthin and fucoxanthin, and affords the best means of effecting a separation of the two classes of carotinoids.

Solutions of carotin in alcohol and the fat solvents show a characteristic absorption spectrum, exhibiting two, and under proper conditions three absorption bands in the green and blue part of the spectrum, the positions of the bands varying somewhat with the refractive index of the solvent. The bands are identical in ether, alcohol and petroleum ether because of the close agreement in the indices of refraction of these solvents, but are shifted somewhat towards the red in chloroform, which has a higher refractive index, and still further away from the blue in carbon disulfide. The marked shift of the bands into the brighter part of the spectrum when in the last named solvent makes it especially useful for observing the spectroscopic properties of carotin, as well as the other carotinoids.

Leaf extracts containing chlorophyll can not be used for a study of the absorption spectra of the carotinoids because the absorption bands of the chlorophylls cover the second and third bands of the carotinoids. Even the first carotinoid band coincides very closely with Band VIII of chlorophyll b.

The width and intensity of the absorption bands of carotin depend

on the concentration of the solution used and the depth of the layer through which the light passes into the spectroscope and thence to the eye of the observer. This fact, together with the fact that the edges of the absorption bands are not sharp and clear cut like the lines of the solar spectrum, no doubt explains the slight differences between the data given by various observers as to the width of the several absorption bands of carotin and the other carotinoids. In spite of this fact, however, the absorption bands of carotin solutions are sufficiently characteristic to distinguish the pigment sharply from the other carotinoids, at least from lycopin and the xanthophylls and rhodoxanthin. Plate 1, showing a spectrophotograph of the bands of carotin and xanthophyll in alcohol and carbon disulfide, brings out this point very clearly, as well as the diffuse character of the edges of the bands. It may be stated, however, that the bands may be somewhat sharper to the eye than is represented in these photographs. The characteristic feature of the bands of carotin which it is desired to point out is that in alcohol (an identical spectrum is obtained in ether and petroleum ether) the solar line F divides the first band almost exactly into two equal parts. This is a characteristic of the first carotin band which may serve to identify the carotin spectrum from that of the other carotinoids.

For direct spectroscopic observations a spectroscope with too wide a dispersion may fail to show any bands in a carotin solution which exhibits very beautiful bands using a spectroscope with a moderate dispersion of the spectrum. In working with unknown biological material the writer has had better success using an inexpensive spectroscope with a moderate dispersion whose spectrum field has been standardized, although arbitrarily, first with the sodium flame and then with known solutions of the carotinoids. Such a spectroscope set up in a dark room with a light of high candle power concentrated on the slit of the instrument but screened from the observer, gives excellent results.

Willstätter and Stoll (1913) give the following measurements for the absorption bands of carotin in solutions containing 5 mg. of pigment per liter, using a grating spectroscope. These data correspond with the spectro-photographs shown in Plate 1.

	Carotin in alcohol (μμ)		*Carotin in carbon disulfide* (μμ)	
	5 mm.	*10 mm.*	*10 mm.*	*20 mm.*
Band I	492–478	492–476	524–510	525–508
Band II	459–446	459–445	489–475	490–474

Kohl (1902b), using a Zeiss spectroscope, obtained the measurements shown in Table 16, using various solvents with different refractive indices. The data also show the bands of solid carotin, obtained by depositing a very thin layer of carotin crystals on one side of a glass slide.

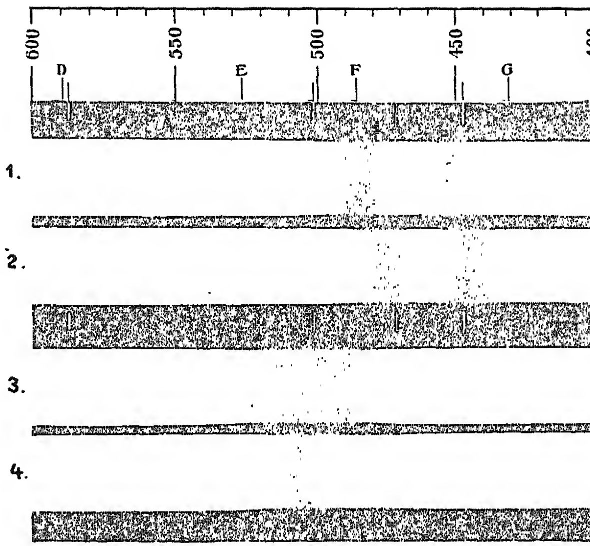
Lycopin. This red isomer of carotin forms yellow solutions in hot ether, chloroform, alcohol, benzene and petroleum ether. These solutions have a somewhat brown tone in comparison with similar solutions of carotin. Even saturated solutions of lycopin in these solvents, with the possible exception of chloroform, contains much less pigment than the corresponding solutions of carotin. This probably accounts for their yellow color. Solutions of lycopin in carbon disulfide are characterized by their bluish-red color which persists even in great dilution, while solutions of carotin in this solvent change to a yellowish red color on great dilution. The effect of the addition of mineral acids to alcoholic solutions of lycopin has not been investigated. Lycopin, however, because of its great oxidizability reacts toward ferric chloride like the other carotinoids. The relation of lycopin toward adsorbents remains to be studied.

Lycopin shows its hydrocarbon nature by exhibiting the same relative solubility properties as carotin when examined by the phase test between petroleum ether and dilute alcohol, on the one hand, and between dilute alcohol and carbon disulfide on the other hand. In each case the pigment is found quantitatively in the petroleum ether or carbon disulfide.

TABLE 16. VISIBLE ABSORPTION SPECTRA OF CAROTIN IN VARIOUS SOLVENTS WITH DIFFERENT REFRACTIVE INDEX (KOHL, 1902b)

Solvent	*Refractive Index*	*Position of bands* (μμ) *Band I*	*Band II*	*Band III*
Alcohol	1.358 (ave.)	490–475	455–445	430–418
Ether	1.357	490–475	455–445	430–418
Acetone	1.365	500–478	460–450	430–420
Chloroform	1.449	505–480	465–450	435–420
Carbon tetrachloride	1.460	507–480	466–452	435–420
Carbon disulfide	1.628	510–485	470–458	437–425
Solid carotin	?	550–530	495–480	460–450

One of the most characteristic properties of lycopin solutions which is especially serviceable for the identification of the pigment is the position of the absorption bands. The relation of the lycopin spectrum in carbon disulfide to that of the other carotinoids in the same solvent is shown in Figure 1, taken from the paper of Monteverde



Spectrophotograph of absorption bands of carotin and xanthophyll in alcohol and carbon disulfide. (After Willstätter and Stoll)

1. Carotin in alcohol
2. Xanthophyll in alcohol
3. Carotin in carbon disulfide
4. Xanthophyll in carbon disulfide

PLATE 1

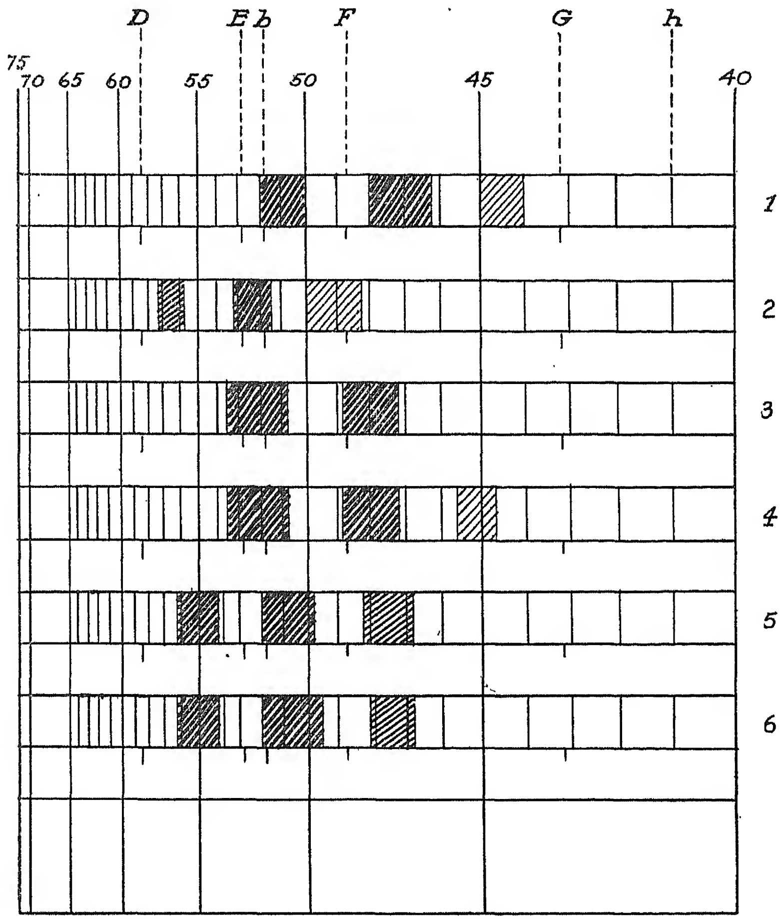


FIG. 1. Relative position of absorption bands of various carotinoids in carbon disulfide solution. (After Monteverde and Lubimenko)

1. Xanthophyll
2. Rhodoxanthin
3. Carotin (according to Willstätter)
4. Carotin (according to Monteverde)
5. Lycopin (according to Willstätter)
6. Lycopin (according to Monteverde)

and Lubimenko (1913b). The lycopin bands represent the general impression which one obtains when viewing a solution containing about 5 mg. per liter at a depth of about 20 mm. The relative positions of the lycopin and carotin bands are very characteristic, but they at once introduce the difficulty that a mixture of the two pigments would show an almost continuous absorption spectrum. It is seen, therefore, that lycopin solutions should be nearly free from carotin in order to identify lycopin by the position of its absorption bands. No means have yet been devised for effecting such a separation when the isomers are present together in solution. Fractional crystallization must be resorted to, and this is made possible by the fact that lycopin is much less soluble than carotin in almost all the carotin solvents.

The measurements of the absorption bands of lycopin in alcohol and carbon disulfide are given by Willstätter and Escher as follows, the bands in carbon disulfide being those shown by a standard solution containing 5 mg. per liter.

	Lycopin in alcohol (μμ)	*Lycopin in carbon disulfide* (μμ)	
		10 mm.	*20 mm.*
Band I	510–499	554–540	561 –536
Band II	480–468	514–499.5	517.5–498
Band III	440–	479–472	481.5–468

Xanthophylls. As pointed out in Chapter II, the chromatographic evidence of Tswett seems to justify the assumption that several isomeric xanthophylls exist in nature, in spite of the fact that only one such pigment has so far been secured in definite crystalline form. Willstätter has not yet agreed to an unqualified acceptance of this assumption. However, if the fact that Tswett's observations can be readily verified is sufficient grounds for accepting his view of the situation, the existence of more than one xanthophyll can no longer be doubted. At the same time it is recognized that we owe most of our knowledge of the properties of xanthophyll solutions to the observations of Willstätter and Mieg (1907), who first isolated pure xanthophyll crystals in sufficient quantity to determine their elementary composition. For the distinguishing characteristics of the other xanthophylls which have not been crystallized it is necessary to refer to the observations of Tswett (1911).

Xanthophylls give well-colored solutions in a large number of solvents, including alcohol, ether, acetone, chloroform, benzene, carbon tetrachloride, glacial acetic acid, petroleum ether, carbon disulfide

and formic acid. Well colored solutions in low boiling petroleum are difficult to secure because of the low solubility of the pigment in this solvent, crystalline xanthophyll being almost insoluble in this solvent. However, xanthophyll in the amorphous state or contaminated with lipoids can be dissolved quite readily even in petroleum ether. The solutions in all these solvents, except carbon disulfide and formic acid, are yellow. These solutions are distinguished from the corresponding carotin solutions by showing a strong greenish tinge on great dilution. The solution in formic acid, which is mentioned only by Monteverde and Lubimenko (1913b), is green. Carotin and lycopin do not dissolve in this solvent. Carbon disulfide solutions of xanthophylls are orange to orange-red, never blood red or bluish red like carotin and lycopin.

The relative color intensities of solutions of carotin and xanthophyll at equi-molar concentrations in different solvents varies considerably with the depth of the solutions. Willstätter and Stoll have compared crystalline xanthophyll and carotin solutions and have obtained the following results.

5×10^{-5} MOLAR SOLUTIONS IN CARBON DISULFIDE

Layer of carotin in mm.	*Layer of xanthophyll in mm.*	*Relative intensity*
12	50	1 : 4.1
25.5	87	1 : 3.4
38.5	120	1 : 3.1
85	180	1 : 2.1

5×10^{-5} MOLAR SOLUTIONS, CAROTIN IN PETROLEUM ETHER-ETHER, XANTHOPHYLL IN ETHER

10	20	1 : 2.0
40	60	1 : 1.5
91	120	1 : 1.3

Xanthophyll can be obtained in aqueous colloidal solution in the same manner that colloidal carotin solution is obtained, according to van den Bergh, Muller and Broekmeyer. Egg yolk and blood serum xanthophyll were used as the source of pigment in the experiments by these investigators.

Only very strong alkali seems to affect alcoholic solutions of xanthophylls adversely. Saponification of xanthophyll solutions with 20 per cent alcoholic potash solutions to remove admixed fat, apparently does not affect the general properties of the pigments. Willstätter and Mieg found, however, that heating amyl alcohol solutions of crystalline xanthophyll with sodium decolorized the pigment, and heating benzene solutions with granulated potassium in an at-

mosphere of hydrogen converted some of the pigment into a product which still retained the solubility of xanthophyll in ether, giving a yellow solution, but which readily formed an ether-insoluble salt with alkali.

Willstätter and Page (1914) state that xanthophyll is incompletely recovered by ether after dissolving in concentrated methyl alcoholic KOH. These facts all point to the possibility of xanthophyll being attacked by alkalis under certain conditions.

The effect of adsorbents on petroleum ether and carbon disulfide solutions of the xanthophylls is especially characteristic and serves not only to distinguish these carotinoids from the hydrocarbon carotinoids but also from each other. Tswett (1906b) has shown that thoroughly dried precipitated calcium carbonate, inulin, sucrose and many other compounds, which are insoluble in petroleum ether, will completely adsorb the xanthophylls when their petroleum ether solution is shaken with an excess of the adsorbent. In order to bring about this adsorption, however, no trace of alcohol must be present, Tswett having shown that petroleum ether containing only one per cent alcohol releases the bulk of the xanthophylls from the adsorbing agent. There is therefore good reason to believe that much smaller amounts of alcohol will interfere greatly with the adsorption.

While this gross test may serve to distinguish the carotinoids containing oxygen from the hydrocarbon carotinoids, the principles involved can also be used to analyze further the xanthophylls and even to separate them from each other. The general principle which is thus utilized is that when several substances present in a single solvent are all adsorbed by a single adsorbent, there is more or less replacement of one adsorbed substance by the others, depending upon the relative affinities of the several substances for the adsorbent, especially if the adsorption compounds in each case are dissociable. This is the principle of Tswett's chromatographic analysis of plant extracts containing chlorophyll and the carotinoids.

The technic which is used for the analysis of a mixture of carotinoid (and chlorophyll) pigments by this method is as follows: A very finely divided adsorbent is selected which will have no oxidizing or reducing or hydrolyzing action on the pigments to be examined. Calcium carbonate is especially recommended. Powdered sucrose is also very suitable. The calcium carbonate is first dried for several hours at 150° C. A glass adsorption tube 1 to 2 cm. in diameter and 10 to 15 cm. long is now prepared which is drawn out at one end.

The small end is left with only a small opening, 1 to 2 mm. in diameter. A plug of cotton is placed in the small end, pressed down tightly and the tube is then filled with the dry $CaCO_3$, which is poured in a little at a time and packed in as tightly and evenly as possible with a glass rod or wooden stick. The success of the chromatograph depends upon the evenness with which the adsorbent is packed into the tube. The tube is filled within 2 or 3 cm. of the top, and a final plug of cotton placed upon it. The tube is now set up through a rubber stopper fitted into a small filter flask, gentle suction applied and a stream of pure solvent (either petroleum ether or carbon disulfide, depending on the solvent selected for the pigment solution) passed through the column until the adsorbent is moistened with it. The suction is stopped and the upper cotton plug removed. Sufficient pigment solution is now poured into the tube to color about 1 cm. of the adsorbent. When this has passed into the column with the aid of gentle suction, the tube is filled with solvent and suction continued. The upper part of the tube is kept filled with pure solvent in order to establish a stream of the solvent through the adsorbing column. The layer of pigment will now pass through slowly and will differentiate itself into zones of relative adsorption, that of greatest adsorption affinity being at the top, and that of the least at the bottom. Inasmuch as all the chlorophylls and carotinoids form dissociable compounds with $CaCO_3$ the stream of pure solvent will slowly wash them through the column as differently colored zones. If the column has been packed perfectly evenly with the adsorbent the zones will be true rings, otherwise they will be irregular. Perfectly true adsorption rings are difficult to secure. Pigments obtained in the various zones by this method are not pure, as Tswett has pointed out, but can be purified by repeating the analysis on the pigment obtained in any desired zone.

A chromatographic analysis applied in the above manner to a petroleum ether or carbon disulfide solution of carotin and the four xanthophylls recognized by Tswett should show the following result. Assuming that carbon disulfide has been used and the differentiation has been continued with a stream of solvent until the least adsorbed pigment has reached the bottom of the column the lowest zone will be rose colored due to carotin; above this, probably separated by a more or less colorless region, will be a wide orange-yellow zone, due to xanthophyll *a*, which apparently comprises the major part of the xanthophylls; still higher in the column and separated from the

xanthophyll α will be a yellow zone due to xanthophylls α' and α'', whose differentiation will be described in a moment; near the top of the column will be a narrow yellow zone, due to xanthophyll β. If a stream of benzene is run through the column at this point, the carotin and xanthophyll α will be quickly washed away and the yellow zone containing xanthophylls α' and α'' will slowly separate into two zones. These can now be washed out of the column with petroleum ether containing one per cent absolute alcohol, leaving xanthophyll β still adsorbed. This pigment can be removed, however, by petroleum ether containing 10 per cent absolute alcohol.

While a chromatographic analysis of an unknown pigment solution is instructive it does not necessarily provide a means of definite identification of any xanthophyll pigments which may be present. Any pigments differentiated by this test must be submitted to further examination. When several pigments are shown by such an analysis, a second chromatographic separation should be carried out on a solution of each of the pigments for the purpose of purifying it as far as possible. Comparison can then be made with the known properties of solutions of xanthophylls α, α', α'' and β, which are as follows:

Xanthophyll α. This pigment is quantitatively removed by 80-90 per cent alcohol, preferably methyl alcohol, from its solution in petroleum ether. It is adsorbed by an excess of $CaCO_3$ from pure, absolutely alcohol-free, low boiling petroleum ether. It is the least adsorbed by $CaCO_3$ from CS_2 of any of the xanthophylls. Its carbon disulfide solutions are orange to red orange. Its alcoholic solution is bleached by addition of concentrated mineral acids, passing through a green color before fading. Its spectroscopic absorption bands are identical with those of crystalline xanthophyll. Plate 1 shows these bands in alcohol and carbon disulfide in comparison with those of carotin. The measurements of these bands, using a solution containing 5 mg. of pigment per liter, are stated by Willstätter and Stoll to be as follows:

	Xanthophyll in alcohol ($\mu\mu$)		*Xanthophyll in carbon disulfide* ($\mu\mu$)	
	5 mm.	*10 mm.*	*10 mm.*	*20 mm.*
Band I	484–472	488–471	515–501	516–501
Band II	454–441	454–440	482–469	483–467
Band III	419–	420–		447–441

Xanthophylls α' and α''. These pigments are quantitatively extracted from petroleum ether by 80-90 per cent alcohol, preferably

methyl alcohol. They are readily adsorbed from petroleum ether by $CaCO_3$ and more readily adsorbed from carbon disulfide by $CaCO_3$ than xanthophyll α. The carbon disulfide solutions are yellow to orange. The pigments are readily released from adsorption on $CaCO_3$ by benzene and when thus adsorbed in a chromatogram may be separated from each other by this solvent. The action of concentrated mineral acids from the alcoholic solution of these xanthophylls is not known, but it may be similar to that on xanthophyll β. The absorption bands of these xanthophylls is stated by Tswett to be shifted slightly towards the violet from those of xanthophyll α. The measurements of these bands has not been reported.

Xanthophyll β. This carotinoid, like the other xanthophylls, is quantitatively extracted from petroleum ether by 80-90 per cent alcohol. It forms almost undissociable adsorption compounds with $CaCO_3$ when in petroleum ether or carbon disulfide, but can be released from this combination by petroleum ether containing 10 per cent absolute alcohol. Concentrated mineral acids produce a green color, passing to a peacock blue when added to its alcoholic solution. NH_4OH will restore the yellow color and acid the blue color. The reaction is similar to one shown by fucoxanthin, in which a hydrochloride is formed, and in which the yellow pigment restored by alkali still retains one molecule of HCl. The absorption bands of alcoholic solutions of xanthophyll β lie at 475-462μμ and 445-431μμ, which are seen to be shifted appreciably towards the violet from the bands of crystalline xanthophyll.

Rhodoxanthin. This red isomer of the xanthophylls is known largely through the properties of its solutions, pure crystals of the pigment not yet having been obtained in sufficient quantity for analysis. This carotinoid forms yellow solutions in petroleum ether, ether and benzene, like other carotinoids, but its alcoholic and acetone solutions are rose colored or pink. It is also dissolved by glacial acetic acid with a red color. The red color in certain solvents serves to distinguish the pigment from other carotinoids, as does also the ruby red or violet red color in carbon disulfide. Formic acid also dissolves the pigment, at first with a pink color which later turns yellow.

Rhodoxanthin, in common with other carotinoids, is not readily attacked by alkali. Its xanthophyll-like character is shown by the fact that 80 per cent alcohol quantitatively extracts the pigment from its solution in petroleum ether. In common with crystalline xanthophyll

the crystals show only very slight solubility in petroleum ether. When its petroleum ether or carbon disulfide solutions are analyzed by means of the chromatograph the pigment shows very little adsorption affinity for $CaCO_3$, its adsorption zone preceding all the others in a chromatographic analysis of extracts obtained from leaves in which the pigment abounds, like the winter foliage of arbor vitæ (*Thuja orientalis*). When carbon disulfide is employed as solvent the rhodoxanthin zone has a characteristic ruby red color.

Solutions of rhodoxanthin show three absorption bands in a characteristic position in the spectrum, being shifted farther towards the red than any of the other carotinoids. The position of the bands, taken from the observations of Monteverde and Lubimenko (1913b), which appear to be the most accurate, are as follows:

	In petroleum ether (μμ)	*In carbon disulfide* (μμ)
Band I	530–513	575–553
Band II	495–480	535–515
Band III	470–455	500–480

The relation of these bands, when in carbon disulfide, to the bands of the other carotinoids in the same solvent is shown in Figure 1.

The effect of mineral acids upon the alcoholic solution of rhodoxanthin has apparently not been determined.

Fucoxanthin. This carotinoid, which is characteristic of the brown algæ, gives well colored solutions in practically all the organic solvents. Although the pure crystals are completely insoluble in petroleum ether, the presence of lipoids makes it possible to obtain colored solutions in this solvent also. This is likewise true of methyl alcohol in which the pure crystals are very sparingly soluble. The ether solution of fucoxanthin is orange yellow, the alcoholic solutions have a somewhat rusty, or brownish yellow tinge, and the carbon disulfide solution is deep red. Fucoxanthin is a more intense pigment than either carotin or crystalline xanthophyll. Willstätter and Page (1914) have stated that a comparison of 5×10^{-5} molar solutions of the three pigments in ether shows that 50 mm. of fucoxanthin is equal in color to 80 mm. of the carotin and 108 mm. of xanthophyll.

The effect of adsorbents on petroleum ether and carbon disulfide solutions of fucoxanthin has not been studied, but the very low solubility of the pigment in petroleum ether suggests that it would be readily adsorbed from this solvent by $CaCO_3$.

Solutions of fucoxanthin show two well defined absorption bands,

but the positions of the bands are not sufficiently characteristic to distinguish the pigment sharply from carotin or xanthophyll. The bands of the alcoholic solution, containing 5 mg. per liter are given by Willstätter and his co-workers as follows:

	Fucoxanthin in alcohol (μμ)		
	5 mm. layer	*10 mm. layer*	*20 mm. layer*
Band I	486–469	492–476	498–473
Band II	455–440	467–451	462–443
End Absorption	440–...	....	....

One of the most characteristic properties of fucoxanthin solutions which can be used as an aid in identification as well as a means of separation of the pigment from other carotinoids is the fact that 70 per cent methyl alcohol will quantitatively extract the pigment from its solution in petroleum ether—ethyl ether (1:1). This fact has already been pointed out in connection with the isolation of the carotinoids, and is especially useful in the quantitative estimation of fucoxanthin as will be shown in the next chapter.

Fucoxanthin solutions are very much less stable than those of the other carotinoids, particularly in the light. Benzene solutions bleach especially readily. Ether solutions of fucoxanthin give a reaction with HCl which resembles in many respects the action of mineral acids on alcoholic solutions of xanthophyll β. When the ether solution of pigment is shaken with 30 per cent HCl solution the pigment bleaches and the acid layer takes on a magnificent blue-violet or sky-blue color. The latter is due to a stable salt containing 4 atoms of HCl, which is probably an oxonium compound. Its solubility in the aqueous layer is due only to the ether which is dissolved in the acid solution. On regeneration of the yellow pigment with alkali, the hydrochloride still persists and retains one atom of HCl. Fucoxanthin apparently unites with other substances as well as HCl for Willstätter and Stoll state that ether solutions dried over $CaCl_2$ yield a pigment showing 3 to 4 per cent CaO.

Another especially characteristic property of fucoxanthin is the action of alkalis on its solutions, or rather on the pigment itself when in solution. The pigment apparently has no acid properties but under certain conditions it is attacked by alkali. Metallic sodium, solid $Ba(OH)_2$ and 50 per cent KOH have no effect upon it. It is dissolved, however, by strong aqueous KOH solutions, and cannot be extracted from this solution by ether. This is also true of con.

methyl alcohol KOH, and in this solvent the pigment is changed so that on dilution with water and extraction with ether (the pigment being liberated from its temporary alkali compound by water) it is much more sensitive towards HCl. Ether solutions will now give the blue color reaction on shaking with only 16 per cent HCl solution, whereas 25 per cent HCl solutions had scarcely any effect before the treatment with alkali. Even 3 per cent HCl now has a noticeable effect, and in concentrated ether solution even 0.001 per cent HCl will give the blue color. The hydrochlorides formed in these cases apparently contain even more chlorine than the hydrochloride which the original pigment forms.

The ether solution of fucoxanthin which has been changed by the concentrated methyl alcohol KOH has a greenish tinge and shows a spectrum whose bands are shifted considerably towards the violet. The ether solution of this pigment containing 5 mg. per liter shows bands at 461-451$\mu\mu$ and 435-423$\mu\mu$ in 10 mm. layer.

Properties of Crystalline Carotinoids

Carotin. Carotin crystallizes from carbon disulfide on addition of absolute alcohol, forming rhombic tablets or prisms, and from petroleum ether, forming almost quadratic leaflets, which are frequently indented. Plate 2, figure 1, shows the form of crystals from carbon disulfide-alcohol. The color of the crystals varies with their thickness from bright yellow to deep rose or copper colored with a rich velvety appearance. The crystals are highly pleochromatic and have an intense blue to bright green metallic luster by reflected light. The crystallography of carotin has been described in detail by Kohl (1902b). Some investigators have ascribed a striking violet or crocus-like odor to the pure crystals, but this has not been observed by others, e.g., Willstätter. The crystals from alcohol usually contain some alcohol of crystallization, which is given up in vacuum over H_2SO_4 or P_2O_5.

Pure carotin crystals are almost insoluble in cold ethyl alcohol, and even less so in methyl alcohol. They dissolve with difficulty in the hot alcohols. About 1.5 liters of low boiling petroleum ether are required to dissolve one gram, under a reflux condenser, but the solubility is somewhat greater in the higher boiling gasoline fractions. About 900 cc. of hot ether are required for one gram of crystals. Acetone dissolves the crystals with difficulty, even hot acetone not

FIG. 1. Carotin from carbon disulfide-alcohol. (× 62)

FIG. 2. Xanthophyll from methyl alcohol. (× 62)

FIG. 3. Xanthophyll iodide from alcohol.

FIG. 4. Lycopin from petroleum ether. (× 165)

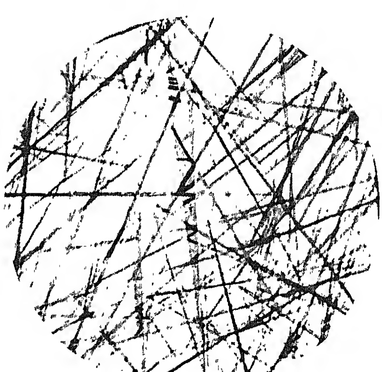


FIG. 5. Lycopin from carbon disulfide-alcohol. (× 165)

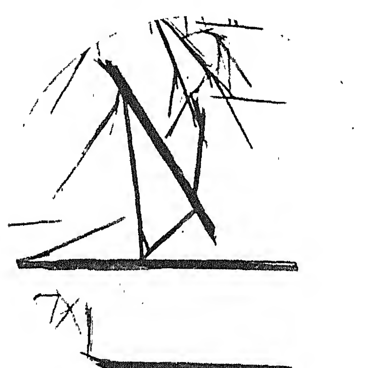


FIG. 6. Fucoxanthin from methyl alcohol.

PLATE 2

being a ready solvent. Benzene dissolves the pure crystals much more easily and chloroform and carbon disulfide with great ease. Kohl (1902e) gives the specific rotation of carotin in chloroform as $\alpha_D = -30.17°$, but this property is not mentioned by other investigators.

Carotin crystals, and even the amorphous pigment, if free from lipoids, dissolve in concentrated H_2SO with an indigo blue color, from which the pigment precipitates as green flakes on dilution. A similar color reaction is given by concentrated HNO_3, dry sulphurous acid and by thymol and phenol containing concentrated HCl. The crystals also give a transient blue color with bromine water and with bromine vapor. With ferric chloride a deep green color is given. This reaction was explained in a previous paragraph. The color reaction of carotin with H_2SO_4, which is also given by the other carotinoids, is regarded by many as a specific reaction for these pigments. There is no justification for this idea, which may easily lead to erroneous conclusions, because this reaction is given by a large number of organic compounds, especially by certain quinones of the aromatic group.

The crystals of carotin readily oxidize, whereby the crystals bleach entirely. The original melting point of 167.5°-168° C. falls and the pigment changes markedly in properties. A number of investigators have reported that the bleached pigment shows the color reactions of cholesterol, but Willstätter and Mieg and Euler and Nordenson were unable to confirm this. The amount of oxygen which carotin is capable of taking up during the oxidation has been variously reported, Arnaud reporting 21 to 24 per cent, Kohl as high as 37.87 per cent. Willstätter and Mieg obtained a maximum of 34.3 per cent, corresponding to 11 atoms of oxygen. Willstätter and Escher (1910) obtained an oxidized product in dry oxygen corresponding to nearly 12 atoms of oxygen. They found that oxidation in a room saturated with moisture gave a product with a like amount of oxygen but containing 2 molecules of water, in addition. The perfectly pure pigment crystals did not oxidize readily. By placing them in a stream of pure oxygen the increase in weight was only 0.3 per cent after five days. After this the oxidation was more rapid, and was accompanied by the violet-like odor which had been described by others for the pure pigment.

Carotin being an unsaturated hydrocarbon would be expected to form stable halogen derivatives. Two iodides have been described, one by

Arnaud (1886) which would correspond to the formula $C_{40}H_{56}I_3$, according to our present accepted composition of the hydrocarbon. This iodide is formed by adding iodine to a petroleum ether solution of carotin crystals in less than the required amount to combine with all the carotin. The iodide crystallizes out as dark violet leaflets with a copper colored reflection, which melt sharply at 136°-137° C. Willstätter and Escher obtained the same iodide by adding double the amount of iodine crystals to benzene-carbon disulfide and carbon disulfide-ether solutions of carotin. The other iodide, described by Willstätter and Mieg and also by Willstätter and Escher (1910), corresponds to the formula $C_{40}H_{56}I_2$, and is prepared by adding crystalline iodine to an ether solution of carotin in an amount equal to only one-third the weight of carotin present. The form and color of these crystals are the same as those of the tri-iodide, but differ from it by showing no definite melting point, the crystals slowly decomposing between 140° and 170° C.

The analyses which have been made of the two iodides, one containing two atoms and the other three atoms of the halogen, show excellent correspondence with the theoretical amount of iodine in compounds showing this composition. It is not clear, however, just what structure of the carotin molecule would permit the formation of an iodide containing three atoms of iodine.

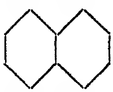
Carotin also forms a bromine derivative which is at once both an addition and a substitution product, which conforms with the constitution $C_{40}H_{36}Br_{22}$. The formation of this product is stated by Willstätter and Escher to take place on the addition of 0.5 grams of powdered carotin in small portions with shaking to 16 grams of bromine at 0° C., the bromine being protected from moisture in the reaction flask by a $CaCl_2$ tube. The reaction is completed by standing at room temperature and the precipitate filtered off on glass wool and washed with hot anhydrous formic acid, giving a brittle colorless product without definite crystalline form. The bromide has no melting point but decomposes at about 171°—174° C. It dissolves easily in benzene and carbon disulfide, fairly easily in ether, but with difficulty in even hot alcohol or petroleum ether; it is insoluble in glacial acetic and anhydrous formic acid. The bromine cannot be completely removed with zinc dust and glacial acetic acid, or with silver acetate.

The structure of the carotin molecule has proved to be a difficult problem to solve. Escher's (1910) attempt resulted only in the production of amorphous products of high molecular weight. One nat-

urally wonders whether any of the known compounds of the same empirical formula bear any relation to carotin, but it must be admitted that they throw no light on its constitution. The simplest empirical formula for carotin, i.e., $(C_5H_7)_8$ suggests a possible relation to the terpene derivatives, the cymenes, or to the toluene derivatives, durene and the propyl toluenes, all of which have the empirical formula $C_{10}H_{14}$. The cymenes, however, are structurally isopropyl benzenes, and durene is tetramethyl benzene. The former is a colorless oil and the latter a colorless solid, m.p., 79-80° C. There seems to be no reason for believing that carotin is related in any way to these substances or to the propyl toluenes.

The problem of the structure of carotin is of special interest because pigmented hydrocarbons are something of a novelty. Those which have been described will be mentioned briefly, inasmuch as their constitution at least indicates the probably chromatophor group in carotin, namely, >C:C<.

Apparently the first pigmented hydrocarbon to be mentioned in the literature is acenaphtylene, $C_{12}H_8$, probably having the structure


HC:CH

. It was first described by Behr and van Dorp (1873) and Blumenthal (1874) and later by Graebe (1893). It forms leaflets of a golden yellow color, soluble in alcohol, ether and benzene, which melt at 92°-93° C. De la Harpe and van Dorp (1875) and later Graebe (1892) have described the hydrocarbon di-biphenylenäthene, $C_{26}H_{16}$, which crystallizes as intensely yellowish red needles and scales, m.p., 187°-188° C. (corrected), and forming yellow solutions. According to Graebe this hydrocarbon adds hydrogen readily, going over into the colorless compound $C_{26}H_{18}$. Thiele (1900a) found that the hydrocarbon fulven, $C_2H_4.C:C.CH_2$ forms brilliantly colored derivatives. He mentions dimethyl fulvene, C_8H_{10}, a bright orange colored oil, methyl phenyl fulvene, $C_{13}H_{12}$, a red oil, and diphenyl fulvene, $C_{18}H_{14}$, which crystallizes from petroleum ether as deep red prisms, m.p., 82° C. Thiele (1900b) has also described the hydrocarbon cinnamylidenindene, $C_6H_5.CH:CH.C — CH:CH$, which crys-

C_6H_5.

tallizes as yellowish-red needles, m.p., 190° C., and is easily soluble in most organic solvents. More recently Pummerer (1912) has dis-

covered the red hydrocarbon, $C_{26}H_{14}$, m.p., 306° C., which he calls rubicen, and which dissolves in $CHCl_3$, giving solutions showing an intense yellow fluorescence. It is not readily soluble in the other organic solvents. The chloroform solution shows one absorption band with a maximum at 498$\mu\mu$. The spectroscopic properties of the other colored hydrocarbons mentioned has apparently not been determined.

Apparently color among hydrocarbons, although rare, is not confined to yellow and red. Sherndal (1915) has isolated a blue hydrocarbon oil, azulene, $C_{15}H_{18}$, from a number of essential oils. It is a coincidence, perhaps worth mentioning, that this blue hydrocarbon dissolves in 60 to 65 per cent sulfuric acid with a yellow color, whereas carotin, a red hydrocarbon, dissolves in strong sulfuric acid with a blue color.

In addition to these substances Marchlewski (1903) called attention to the fact that pigmented compounds could be made starting with methyl-ethyl maleic acid anhydride, which show a strong resemblance to the lipochromes both with respect to spectroscopic properties and color reactions. Marchlewski's note on the subject was for the purpose of reserving the field of investigation, but so far as the writer is aware no further results have ever been published.

Xanthophyll. The crystals of xanthophyll obtained from plants by Willstätter and Mieg (1907) and from egg yolk by Willstätter and Escher show complete correspondence in form, color, solubility, oxidation products and halogen derivatives, but not in melting point. The latter point was discussed in Chapter VI.

Xanthophyll appears to crystallize best from alcohol, preferably methyl alcohol, from which the forms are mostly quadratic, often trapesium tablets, frequently showing indentations. Their general appearance is shown in Plate 2, Figure 2. From ethyl alcohol the crystals are lance- and wedge-shaped prisms. Sometimes the crystals are rhombic, almost hexahedrons. In all cases the crystals contain a molecule of the solvent from which crystallization occurred. A single example is reported by Willstätter and Mieg in which crystals from CS_2 contained 22 per cent sulphur.

The color of the crystals varies with the thickness from a greenish-yellow to a rose, similar to the crystals of carotin but distinguished by a less red color. The crystals are even more strongly pleochromatic than carotin, their brilliant steel blue reflection being especially evident when suspended in the solvent. The powdered crystals have a brick red to red-lead color. After removing the sol-

vent of crystallization by drying in vacuum over H_2SO_4 or P_2O_5 xanthophyll crystals from plants melt at 173.5°-174.5° C. (corrected), but according to Willstätter and Escher xanthophyll from egg yolk melts at 195°-196° C. (corrected).

Xanthophyll crystals are entirely insoluble in low boiling petroleum ether. The solubility in cold methyl alcohol is quite low, 1 gram requiring about 5 liters, but is considerably greater in the boiling solvent, 1 gram requiring 700 cc. to 1 liter. The solubility of the pure pigment in ethyl alcohol is considerably greater. The crystals dissolve rather easily in warm CS_2, 1 gram requiring about 400 cc. of solvent. The solubility in ether is a little greater and in acetone and chloroform quite rapid. Phenol also dissolves the crystals quickly as does hot glacial acetic acid. A mixture of 3 parts phenol crystals and 1 part glycerol also dissolve xanthophyll crystals very readily, as van Wisselingh (1915) has shown.

Xanthophyll crystals, like carotin, dissolve in con. H_2SO_4 with a deep blue color, from which green flakes are precipitated on dilution with water. They also dissolve in warm ethyl alcohol containing strong HCl with a pure green color which soon changes to blue. This reaction is apparently peculiar to xanthophyll in contrast with the hydrocarbon carotinoids. According to van Wisselingh xanthophyll crystals can be distinguished from carotin crystals by the fact that the former are colored blue but are not dissolved by 65 to 75 per cent H_2SO_4 while the latter turn blue only after some lapse of time or when stronger acid is used. With con. HNO_3 a colorless solution only is obtained from which colorless flakes separate. This is Willstätter and Mieg's finding, but van Wisselingh found that the blue color reaction resulted with 50 per cent acid. This may also serve to distinguish the crystals of the two types of carotinoids.

Xanthophyll, like carotin, is unsaturated and forms an iodide, $C_{40}H_{56}O_2I_2$, which precipitates at once on addition of iodine to the ethereal solution of the pigment. An excess of iodine prevents the crystallization. The iodide has a dark violet color and consists of tuft-like prisms, the form of which is shown in Plate 2, Figure 3. The compound is not very stable and possesses no definite melting point. It is fairly readily soluble in the xanthophyll solvents, excepting ether, giving yellow to yellowish-red solutions. When xanthophyll is brominated it loses its oxygen, since Willstätter and Escher (1910) report a xanthophyll bromide with the constitution $C_{40}H_{40}Br_{22}$.

Xanthophyll crystals slowly oxidize in the air or in oxygen, with

the addition of 36.5 per cent of their original weight, corresponding to 13 atoms of oxygen or the formula $C_{40}H_{56}O_{15}$. When this product is recrystallized from ether it contains even more oxygen and corresponds to the formula $C_{40}H_{56}O_{18}$, and melts at 140° C. The oxidizing pigment has a peculiar violet-like odor, at least in the case of plant xanthophyll, although Willstätter and Escher did not notice this in the case of xanthophyll from egg yolk. The oxidized crystals dissolve in concentrated mineral acids with a dark brown color and in dilute alkalis with an intense reddish-yellow color.

The constitution of xanthophyll, like that of carotin, is unknown. Even its relation to carotin is very puzzling. While the empirical relations between the two carotinoids suggest that xanthophyll is a simple oxidation product of carotin, the behavior of xanthophyll shows that this is not the case. Xanthophyll fails to give a reaction for carbonyl, alcohol or acid groups, which suggests that the oxygen must be present in an ether-like combination. If this be accepted as probable it would indicate that the carotinoids are not derived from each other but are rather built up from a common nucleus.

Lycopin. This red isomer of carotin crystallizes in the form of a bright or dark carmine colored, velvety appearing mat of wax-like crystal aggregates, consisting of elongated microscopic prisms, whose ends are usually quite ragged. The crystals usually obtained from petroleum ether are of this character and are shown in Plate 2, Figure 4. Figure 5, Plate 2 shows the fine needles which crystallize from ether or from carbon disulfide-alcohol, which frequently occur in beautiful starlike clusters, according to Monteverde and Lubimenko. The powdered crystals have a dark reddish-brown color and melt at 168°-169° C. (corrected).

Lycopin crystals are less soluble than carotin in all the carotinoid solvents. Ethyl and especially methyl alcohol are exceptionally poor solvents. Low boiling petroleum ether dissolves only a small amount, 10 to 12 liters taking up only 1 gram. About 3 liters of ether are required for the same amount of pigment, but one can readily obtain a 2 per cent solution in CS_2, and even stronger solutions in warm chloroform or benzene. The crystals are insoluble in acetone and glacial acetic acid. They dissolve, however, in concentrated H_2SO_4 and HNO_3 with a deep blue or purple color, which is very transient in the case of HNO_3.

The lycopin crystals readily oxidize with bleaching, the maximum oxygen absorption amounting to about 32.5 per cent of their original

weight. The oxidizing pigment has a peculiar odor, which is stated by Willstätter and Escher (1910) to be different from that of oxidizing carotin or xanthophyll, but is not described.

When lycopin crystals are dissolved in a little CS_2 and much ether and treated with one-third their weight of iodine, a lycopin iodide corresponding to the probable formula $C_{40}H_{56}I_2$ precipitates in the form of dark green, gelatinous flakes, containing 34-37 per cent iodine. When the lycopin crystals are treated with a trace of bromine vapor they first turn a vivid green, and can then be dissolved in an excess of bromine to form a colorless, resinous material, insoluble in anhydrous formic acid, whose constitution is somewhat difficult to understand in the light of the analyses made by Willstätter and Escher. The combined addition and substitution compound appears to have the constitution $C_{40}H_{44}Br_{26}$, indicating substitution of 12 hydrogen atoms and addition of 14 bromine atoms. This would indicate a much greater instability of the lycopin double bonds than is the case with carotin which forms the bromide $C_{40}H_{36}Br_{22}$, which shows the addition of only two atoms of bromine and corresponds to the di-iodide which the pigment forms.

Fucoxanthin. This carotinoid crystallizes from concentrated methyl alcohol in the forms of long amber colored prisms, belonging to the monoclinic system. The crystals are shown in Plate 2, Figure 6. The powdered crystals are brick red. These crystals contain three molecules of methyl alcohol. When freed of the solvent by desiccation, the crystals are hydroscopic. This water is difficult to remove, being given up only at 105° C., under diminished pressure. Fucoxanthin crystallizes from dilute alcohol or acetone in characteristic hexagon-shaped tablets, containing 2 molecules of water of crystallization. The addition of water to the alcohol or acetone solution of fucoxanthin precipitates the pigment as needles which rapidly change to the hexagon-shaped hydrates. The anhydrous crystals melt at 159.5°-160.5° C. (corrected), those containing methyl alcohol about 10° lower.

Fucoxanthin resembles xanthophyll in its solubilities. One hundred grams of boiling methyl alcohol dissolve 1.66 grams of pigment, but only 0.41 grams at 0° C. The crystals are fairly difficultly soluble in ether, fairly easily in CS_2, and easily in ethyl alcohol. The pure crystals, either hydrates or methyl alcoholates, do not readily oxidize, but the solutions readily bleach, and a product can be obtained from these colorless solutions which corresponds. to the formula $C_{40}H_{54}O_{16}$ or $C_{40}H_{54}O_{14}$.

Fucoxanthin crystals correspond with the other carotinoids in dissolving in concentrated H_2SO_4 and HNO_3 with a blue or purple color, and in addition readily form a blue oxonium salt with HCl, as has already been described. The pigment also forms an iodide, but apparently one containing 4 atoms of iodine, instead of di-iodide which the other carotinoids form. This product is obtained in the form of dark violet, short, pointed prisms with a copper luster, which have a gray to blue-green color by transmitted light when viewed under the microscope. One obtains these crystals by adding the iodine to an ether solution of the pigment. The crystals are easily soluble in chloroform and acetone with a deep blue color. They melt at 134°-135° C. A bromide of the pigment does not appear to have been made.

Methods of Identification in Biological Products

It is at times very desirable to be able to identify carotinoid pigments in the plant or animal tissues in which they occur. So far as plant tissues are concerned it is possible to make a gross identification of carotinoids with certainty and even to differentiate carotin and xanthophylls and lycopin with a reasonable degree of accuracy. These results are made possible because of the excellent researches of Molisch (1896), Tammes (1900) and particularly van Wisselingh (1915). A similar identification of carotinoids in animal tissues has not yet been devised; at least it will be pointed out presently how insecure the foundation is upon which the demonstration of animal lipochromes (undoubtedly carotinoids) has been built. Attention will be directed first to the possibilities in connection with plant tissues.

Plant tissues. The demonstration and identification of carotinoids in the plant tissues in which they are formed rests upon a microchemical crystallization of the pigments in the tissues and a study of the effect of certain solvents, and reagents producing color reactions, upon these crystals. As has already been pointed out in a previous chapter, carotinoids occur almost entirely in the plastids in plant tissues and very rarely as crystals in the cells. As van Wisselingh has stated, the carotinoids occur mostly bound to fluid, fat-like, saponifiable substances or actually dissolved in them. These substances are in the plastids or they form oily drops in the cells. It becomes necessary, therefore, first to set the pigments free from their union or solution in the plastids. It has been shown by van Wisselingh that of the

methods which have been proposed for the microchemical crystallization of the carotinoids only one can be depended upon to assure this result, namely the alkali method of Molisch. He has shown that it is possible by this method to secure the microcrystallization of all types of known carotinoids occurring in plants, with the possible exception of fucoxanthin. While van Wisselingh found that the brown algæ give excellent crystals it is not clear whether these are fucoxanthin or the carotin and xanthophyll which accompany it in these plants.

The method is carried out as follows. Several small pieces of plant tissue or sections of the same (leaf, petal, slice of fruit or other bulky tissue) are placed in 100 to 200 cc. of alcoholic potash containing 40 per cent alcohol by volume and 20 per cent KOH by weight. The tissue and solvent are placed in darkness protected from the air and allowed to stand until the substances associated with the carotinoids have dissolved or become saponified and the carotinoids present have crystallized out. The time of crystallization will vary with the object from minutes to months but can be greatly speeded up for the latter cases by warming the preparation for a few hours at 70°-80° C., on several successive days. Van Wisselingh has shown that crystals can be obtained in a few days by this modification which may require over a year at room temperature. A piece of the tissue being studied is withdrawn from time to time for examination as to the progress of the reaction. It is first washed thoroughly with water and finally allowed to rest in distilled water for several hours before preparing the section for microscopic examination.

When carotin and xanthophylls are present the crystals which will form divide themselves into two general classes according to their color, one group, probably due to xanthophylls, being orange-yellow to orange and the other, probably due to carotin, being orange-red to red. It is not safe, however, to depend upon the color of the crystals for determining their character, because rhodoxanthin, if present, would undoubtedly crystallize in the red group. In fact, van Wisselingh encountered red xanthophyll-like crystals in his investigation.

Lycopin does not form microcrystals in the Molisch method. A slight modification, however, permits their formation, namely, heating the tissue to 140° C. in glycerol alone or in glycerol containing 10 per cent KOH. Lycopin forms reddish violet microscopic crystals under these conditions.

The formation of crystals in plant tissues by the methods described is alone sufficient for a gross identification of carotinoids. For verification, however, the crystals may be treated either with strong H_2SO_4 or bromine water or with a solution of $SbCl_3$ in 25 per cent HCl. In each case the reagent will impart a blue color to carotinoid crystals of all types. When using the antimony reagent the preparation must first be placed in dilute HCl. In the other cases the preparation may be placed in a minimum amount of water.

The differentiation of the types of crystals as xanthophyll, carotin or lycopin, rests upon two general tests which are reasonably accurate. (1) Xanthophyll crystals dissolve very quickly in a phenol-glycerol mixture made up of 3 parts by weight of phenol and 1 part by weight of glycerol, while carotin and lycopin dissolve very slowly if at all. Van Wisselingh found that carotin crystals from carrots and lycopin crystals from tomatoes remained untouched by this reagent even after several days. (2) Xanthophyll crystals give a quick blue color when treated with 75 per cent H_2SO_4 while carotin and lycopin crystals require a stronger H_2SO_4 to color their crystals blue, or at least to do so quickly.

Animal tissues. The possibility of a microchemical demonstration of carotinoids in animal tissues rests at the present time on the assumption that the methods which have been used for identifying lipochromes in such tissues are in reality methods for detecting carotinoids, and are, moreover, specific for these pigments. Let us see whether these assumptions are justified.

Two methods have been used rather generally for detecting lipochromes in sections of animal tissues. One has been the application of the so-called specific color reactions with concentrated H_2SO_4 and HNO_3 and with iodine-potassium-iodide solution; the other has been the reaction of the pigments towards certain fat stains, particularly Scarlet Red, Sudan III and osmic acid.

Carotinoid pigments are encountered in animal tissues both as intracellular and intercellular substance, generally in more or less granular or amorphous condition but also coloring what appears to be true fat globules. The possibility is also not excluded that they may occur in the tissues bound to protein as they at times occur in the blood. Since carotinoids dissolve readily in liquid fats, one may also expect to find fats at times dissolved in carotinoids. It is therefore an open question whether true lipochromes (carotinoids) ever occur in animal tissues in a pure condition. Since lipochrome is never encoun-

tered in a crystalline condition in such tissues, as it has been stated to occur in plants, the balance of the argument is at present against the occurrence of the pure pigments.

With regard to the lipochrome color reactions the results which have been secured are not encouraging as to their applicability to the carotinoids which occur in animal tissues. The chemistry of the blue color reaction with con. H_2SO_4, which is given by a number of aromatic substances besides the carotinoids, is not known. A positive reaction with this reagent can not therefore be regarded as conclusive proof of the presence of carotinoids, although it would indicate this possibility. This reaction, however, fails completely in the presence of glycerides, and may be vitiated even by the presence of other lipoids which are attacked by strong sulfuric acid. It can not be used at all for detecting carotinoids dissolved in fats. A negative test on even more suitable material is not necessarily conclusive; for example, Sehrt (1904) discredited the corpus luteum pigment as a lipochrome because it was colored only a faint blue by H_2SO_4, and sometimes not at all. The reaction with H_2SO_4, therefore, has only a very limited application to carotinoids which may be encountered in histological sections of animal tissues, at least under the conditions in which it has been employed up to the present time.

Practically the same conclusion must be drawn for the use of the iodine reaction. The cause of this reaction is the blue iodine derivative which is formed by all the known carotinoids. The reaction was discovered by Schwalbe (1874) as an apparently typical pigment reaction for the colored oil drops in the retina of certain animals. Since that time it has been generally held that animal pigments which fail to give the iodine reaction are not typical lipochromes. However, even this reaction, which possesses a firm chemical basis, has frequently failed for pigments which we now know to be true carotinoids. For example, Kühne (1878) failed to secure the reaction with egg yolk pigment, even after isolation, and Sehrt (1904) found that the corpus luteum pigment only occasionally reacted.

These results seem very discouraging. We have already seen, however, that no great difficulty attends the use of color reactions in the microchemical identification of carotinoids in plant tissues. The author is not aware of any attempt to apply the Molisch microchemical method of crystallization of carotinoids to animal tissues. Unfortunately the high concentration of alkali in the Molisch reagent

would undoubtedly disintegrate animal tissues before the pigment could crystallize out. It would be well worth the effort, however, if a microchemical crystallization method could be devised which would be applicable to animal tissues.

The conception of a constant association of pigment with fat which is suggested by the term lipochrome was no doubt in a measure responsible for the introduction of fat stains for demonstrating the presence of such pigments in animal tissues. The writer has not made a thorough study of the history of the use of this technic, since the matter is not of great importance. It is, therefore, a little difficult to state whether the use of fat stains began with the idea of demonstrating that a pigment in question was actually associated with fat and therefore a true "lipochrome," or whether their use was suggested solely by the term itself or by the statements encountered here and there in the literature on plant lipochromes that one of the fat stains (usually osmic acid) imparted its characteristic color to the pigment. Whatever the origin of their use may have been it is obvious that the present conception of the action of the fat stains, as shown by consulting the modern handbooks on biochemistry and pathology, is that they stain the pigments themselves. For example, Wells (1918) states that the lipochromes "are characterized by staining by such fat stains as Sudan III and Scarlet Red, and usually, but not constantly, by osmic acid"; and Herxheimer (1913) makes practically the same statement, without, however, making any reservations with respect to osmic acid.

As far as the true carotinoids are concerned this conception rests upon the uncertain assumption that these pigments are actually stained by such dyes as Sudan III, Scarlet Red and osmic acid. It is possible that such is the case, but unfortunately the matter has never been subjected to an experimental study; and until we have further proof of the action of these and other fat dyes upon the pure pigments it is not possible to state definitely that a positive stain with a fat dye is a positive test for pigment of the carotinoid (lipochrome) type. In fact, there is evidence which indicates that a positive stain with a fat dye is merely a test for the lipoid with which the pigment is associated.

Neumann (1902) states that when the fat cells of the bone marrow and sex glands of frogs have become completely atrophied through inanition (or during hibernation) the lipochrome which remains no longer takes the osmic acid stain, but still gives the reaction with

iodine. Dolley and Guthrie (1919) have studied carotinoids in animal tissues by means of fat stains with results which bear on this question. They observed that amorphous deposits of what appeared to be pure lipochrome in both animal and plant (carrot) tissues still stained with Sudan III and Scarlet Red after the pigment granules had become bleached through oxidation. This fact does not prove that the pigment granules were not pure pigment, but it does throw doubt upon this conclusion. Kreibich (1920) takes the view that the lipochromes in animal tissues, which he calls sudanophiles, are united with alcohol insoluble lipoids.

Dolley and Guthrie (see also Palmer and Kempster (1919b)) made the interesting observation that Nile blue (used either as the hydrochlorate or sulfate in 1-10,000 aqueous solution or as a stronger solution in 65 per cent acetone) when used as a progressive stain, "particularizes the lipochrome first as a deep blue," but stains neutral fat a salmon pink even in the presence of lipochrome. The blue stain was found to occur for the amorphous carotin granules in the frozen carrot section, and for the amorphous xanthophyll granules and minute pigment globules in the stratum corneum of the chicken skin, while the salmon pink color occurred for lard stained deeply with carotin from carrots, for chicken fat highly colored with xanthophyll, for the globules in colostrum milk fat, deeply colored with carotin, and for the fat globules in the natural emulsion of the deep yellow (xanthophyll colored) yolk of the hen's egg. Dolley and Guthrie later (1921) found that the lipochrome granules of the heart muscle stained blue with the dye, although in their earlier (1919) work they were unable to secure positive differentiation of lipochrome and fat in nerve cells by this method.

These findings appear, at first sight, to be a definite advance in the technic of demonstrating carotinoids in animal tissues. This conclusion is weakened, however, by the fact that Smith (1907) showed that Nile blue differentiates fatty acids from neutral fats in the same manner that it appears to differentiate carotinoids from neutral fats, fatty acids staining blue and neutral fat red.[1] This fact alone, however, would not necessarily disprove the supposition that carotinoids may act like fatty acids towards Nile blue, although it must be admitted

[1] Herxheimer (Abderhalden's Handbuch der Biologischen Arbeitsmethoden, *viii*, part 1, 208) states that this fact has been confirmed by Escher (Korrbl. f. Schweizer Ärzte, *49*, 1919) and by Boeminghaus (Ziegler's Beitrage, *67*, 532, 1920) who found also that cholesterol esters of fatty acids stain red like neutral fat, and many other lipoids take a mixed blue and red color.

that the chemistry of the blue stain with Nile blue argues against this supposition. As stated by Smith the simultaneous staining of fatty acids and neutral fat by Nile blue is due to the fact that this dye is a mixure of a strongly basic oxazine which reacts readily with fatty acids to form blue soaps, and a weakly basic oxazone which dissolves readily in neutral fats and fat solvents. When it is remembered that none of the carotinoids have acid properties it may be argued that the blue stain imparted to carotinoid pigment granules in the writer's and in Dolley and Guthrie's experiments merely indicates the acid character of the lipoid with which the pigment was associated. This argument would have to be accepted as conclusive if the oxidized pigment granules should be found to retain their property of taking the blue stain with the Nile blue oxazine like they have been observed to do with Sudan III and Scarlet Red. On the other hand, if it should be found that the oxidized pigment granules in plant or animal tissues no longer take the blue stain with Nile blue there would be strong basis for believing that the oxazine base in the dye is specific for carotinoids as well as for fatty acids. Certainly it would not be unreasonable to assume that the profound changes which undoubtedly occur in the chemical characters of the carotinoids during their oxidation also alter their relation towards dyes.

Hueck (1912) has stated that lipochrome in animal tissue still stains blue with Nile blue after oxidation with hydrogen peroxide. At the writer's suggestion Dr. Dolley has investigated this point more exhaustively using frozen sections of carrot tissue, with the result[2] that Hueck's observation is confirmed. Complete oxidation with hydrogen peroxide and sunlight or careful oxidation with ferric chloride fails to destroy the ability of the visible, bleached pigment granules to take the blue oxazine base from the Nile blue dye. It thus appears impossible to differentiate between carotinoid pigment and fatty acids, although these can be distinguished from neutral fat or esters by the Nile blue dye.

The effect of ferric chloride on the carotinoids is alone of some value in indicating the presence of carotinoids in animal tissues. While it is not possible to obtain the green color reaction when working with tissue sections, on account of the fact that the color reaction is in the reagent itself, pigment granules which are readily oxidized (bleached) by this reagent may be suspected to be carotinoid in nature. Dolley and Guthrie found that a strong solution of ferric

[2] Personal communication.

chloride in 50 per cent alcohol was especially suitable for this purpose. Treatment of sections showing carotinoid granules with hydrogen peroxide for 24 to 48 hours apparently effected the same result, but Dr. Dolley's recent communication indicates that the oxidation is not so thorough as with the ferric chloride unless the peroxide is supplemented with strong sunlight. There is also the tacit assumption among pathologists that sections which give up their pigment to fat solvents contain lipochromes. This, of course, can only be considered contributory evidence..

Summary

It is impossible to summarize adequately in a few words the facts presented in this chapter describing in detail the chemical and physical properties of the several carotinoids both in crystalline form and also when in solution in various solvents.

It may be pointed out, however, that the properties of impure solutions of the individual carotinoids when freshly prepared are sufficiently characteristic for their identification without resorting to the tedious process of isolating the pigments in pure crystalline form. The characteristic properties which may be employed for this purpose include color, spectroscopic absorption bands, relative solubility in alcohol and petroleum ether and adsorption affinity towards finely divided agents like $CaCO_3$.

It is fortunately possible to identify carotinoids in general in plant issues through a microchemical crystallization method. It is possible, also, to roughly differentiate these crystals into groups, such as carotin, xanthophyll and lycopin-like pigments, by means of the effect of a phenol-glycerine solvent on the crystals and the rapidity with which they respond to a color reaction with sulfuric acid of different strengths.

The microchemical demonstration of carotinoids in animal tissues does not rest on a very adequate basis. Recent work, however, indicates that although it is not possible to differentiate between carotinoid pigment and fatty acids, these can be distinguished from neutral fat and esters by means of their characteristic staining reaction with Nile blue, the former staining blue and the latter some shade of pink.

Chapter X

Quantitative Estimation of Carotinoids

The small amount of carotinoids in plant and animal tissues, together with the difficulty of securing the pigments free from colorless impurities as well as the great ease with which the pigments oxidize, forbid their quantitative estimation by a gravimetric method. The great intensity of the carotinoid pigments and their ready solubility in certain organic solvents naturally suggests the possibility of their quantitative estimation by colorimetric methods. The methods which have been proposed have, in fact, been devised on this basis.

Estimation of Carotin and Xanthophyll

Arnaud (1887) was the first to propose a colorimetric method for the quantitative estimation of carotin in plant tissues. The method was based on his observation that air dried, or especially vacuum dried leaves (leaves dried in an oven even at low temperature cannot be used, according to Arnaud) do not give up any of their chlorophyll when allowed to remain in contact with low boiling petroleum ether, but permit all the carotin to be extracted. A further essential feature of his method was based on the observation (a single experiment only is reported) that the color of carbon disulfide solutions of carotin is directly proportional to the amount of carotin present. With these observations as a basis Arnaud proceeded as follows.

Twenty gram quantities of air-dried or vacuum-dried, powdered leaves were shaken up with 1 liter of cold petroleum ether in a stoppered flask for a period of 10 days. The extract was filtered off and exactly 100 cc. evaporated to dryness. The residue was taken up in exactly 100 cc. of carbon disulfide and compared in a Dubosque colorimeter with a standard 0.001 per cent solution of carotin in carbon disulfide. It is stated that the colorimeter was modified slightly to prevent the volatilization of the solvent and that blue glasses were inserted to improve the sensitiveness of the instrument, but the details in regard to these modifications are not given. The data which

Arnaud reports indicate that he set his standard carotin solution at 19 mm. depth and compared his unknown solutions with this arbitrary standard.

Arnaud (1889) used this method for the quantitative analysis of the carotin content of the air-dried leaves of a large number of plants. These data are shown in Table 17. Arnaud expresses his confidence in their accuracy within 1 to 2 mg. of carotin for 100 grams of dried leaves. The principal criticisms which can be made of this method are, (1) the necessity of having a supply of pure carotin on hand for making up the standard solution, (2) the failure to show that the method insures the complete extraction of the carotin, (3) the possibility that some xanthophyll may be extracted along with the carotin, and (4) the necessity of evaporating the petroleum ether extracts to dryness before dissolving in the standard solvent, this operation greatly enhancing the opportunities for oxidation and loss of pigment.

TABLE 17. CAROTIN CONTENT OF AIR-DRIED LEAVES (ARNAUD'S METHOD)

Name of plant	*Date of sample*	*Carotin content mg. in 100 gms.*
Rape (*Brassica oleifera*)	June 1	189.7
Violet (*Viola odorata*)	May 23	124.0
Linden (*Tilia platyphylla*)	May 11	79.1
Maple (*Acer pseudo-platanus*)	June 15	190.0
Sycamore (*Acer platanoides*)	June 15	178.0
Grape (*Vitis vinifera*)	July 12	200.0
Wild grape (*Cissus quinquefolia*)	May 25	145.4
Chestnut (*Aesculus hypocastanum*)	May 6	118.8
Bean (*Phaseolus vulgaris*)	June 18	178.8
Pea (*Pisum sativum*)	May 27	177.0
Acacia (*Robinia pseudo-acacia*)	June 8	209.0
Peach (*Persica vulgaris*)	June 15	114.0
Red currant (*Ribes rubrum*)	May 21	105.5
Ivy (*Hedera Helix*)	May 15	50.9
Periwinkle (*Vinca Major*)	May 25	130.0
Olive (*Olea Europaea*)	July 16	75.0
Potato (*Solanum tuberosum*)	July 21	190.0
Tobacco (*Nicotinia tabacum*)	Aug. 4	178.8
Stramonium (*Datura stramonium*)	July 20	177.0
Spinach (*Spinacia inermis*)	June 1	160.0
Beet (*Beta vulgaris*)	July 12	183.0
Hemp (*Cannabis sativa*)	June 18	215.9
Box tree (*Buxus sempervirens*)	June 4	86.9
Stinging nettle (*Urtica dioica*)	May 2	171.7
Walnut (*Juglans regia*)	May 19	118.8
Yew tree (*Taxus baccata*)	June 4	167.6
Wheat (*Triticum vulgare*)	June 4	167.6
Grass (*Lolium perenne*)	April 18	106.3
Fern (*Pteris aquilina*)	June 1	116.8

Arnaud made an interesting study of the seasonal variations in the carotin content of green leaves, using the stinging nettle and chestnut leaves as the source of his material. He found that the maximum carotin content (on the dry basis) occurred at the time of the flowering of the plants, and that it diminished regularly with the growth of the leaves. Thus, in the case of the nettle, a carotin content of 172 mgs. per 100 grams of dried leaves was noted on May 2, but this had decreased to about 100 mg. by the middle of July. In a comparison between etiolated and green leaves of the same plant, Arnaud found that the carotin content, on the dry matter basis, increased about 5 times during the formation of the chlorophyll.

Kohl (1902i) used Arnaud's method for determining the carotin content of the leaves of a few plants, but secured somewhat lower results. His values for spinach and stinging nettle leaves were about half those reported by Arnaud, and for grass about 70 per cent of Arnaud's value. These low results may have been due, however, to the fact that Kohl apparently ignored Arnaud's precaution and dried his material at 100° C.

Monteverde and Lubimenko (1913a) have devised a spectro-colorimetric method for the quantitative estimation of carotin, as well as xanthophylls and chlorophyll, in green leaves. The writer has not been able to secure a clear translation of this method which has been published only in Russian. In general, however, the method appears to be based on the extraction of all the pigments from fresh leaves, 0.1 gram quantities, by grinding in a mortar with alcohol. Measured quantities of the extract are then treated with strong $Ba(OH)_2$ solution to throw down all the pigments. After standing for some hours, the precipitate is filtered off and extracted completely with absolute alcohol, which is said to take out only the carotinoids. These are fractionated by the Kraus method between 80 per cent alcohol and petroleum ether, and these fractions compared in the spectro-colorimeter with standard 0.001 per cent carotin and xanthophyll solutions. By keeping all extracts in definite volumes the data can be calculated back to the quantity of pigments in the plant tissues examined. The feature of the spectro-colorimetric method is the comparison of the solutions on the basis of the depth of unknown solution required to give an absorption spectra of equal intensity as the standard. The authors found that the first faint appearance of absorption bands for the standard solutions gave a more sensitive compari-

son than stronger bands, and that this was secured at a depth of 3 cm. for the carotin and xanthophyll standards.

Using the above method Monteverde and Lubimenko determined the carotin (and xanthophyll) content of a number of plants. The data are given in Table 18. The results are striking in so far as the low content of carotin is concerned in comparison with the results secured by Arnaud. The writer is not convinced that absolute alcohol will give a quantitative extraction of carotin from the baryta-chlorophyll complex obtained by this method. The data in Table 18 are of interest also in showing quite wide variations between the relative amounts of carotin and xanthophyll in the different plants.

TABLE 18. CAROTINOID CONTENT OF DRY GREEN LEAVES (LUBIMENKO'S METHOD)

Name of plant	*Carotin content mg. per 100 gms.*	*Xanthophyll content mg. per 100 gms.*
Thuja orientalis (arbor vitæ)	20.8	131.7
Viburnum Tinus	47.9	154.3
Luffa gigantia	61.5	354.6
Albizzia Julibrissin	66.7	280.9
Ruta graveolens	94.4	387.6
Ailanthus glandulosa	72.7	263.3
Clematis vitalba	100.6	406.5
Hyssopus officinalis	108.1	355.6
Rubus caesius	106.0	396.8
Arundinaria japonica	106.1	350.0

Willstätter and Stoll (1913) have described in great detail a colorimetric method for the quantitative estimation of carotin and xanthophylls which appears to give very accurate results. The method as given is intended to be used for green plant tissues. For convenience in understanding the details the method may be divided into several parts, as follows: (1) Preparation of the material, (2) extraction of the pigments, (3) removal of the chlorophyll, (4) separation of the carotinoids, (5) colorimetric comparison of the carotinoids with standard solutions.

Preparation of the material. Forty grams of fresh leaves are placed in a mortar (diam. 25 cm.) with 50 cc. of 40 per cent acetone and macerated quickly with 0.5 gram of quartz sand. One hundred cc. of 30 per cent acetone are then poured over the apparently dry mass and the whole mixed for a few minutes. The extract and leafy material are then transferred to a suction filter containing a thin layer of talc, and the extract sucked away. After sucking dry, the material on the filter is washed with 100-200 cc. of 30 per cent acetone in small

portions, or until the filtrate is colorless. According to Willstätter and Stoll the grinding and preliminary extraction require 15 to 30 minutes. The preliminary extracts are discarded.

Extraction of the pigments. The dry mixture of leafy material and sand on the filter is carefully loosened with a spatula and macerated for a few minutes with a small amount of pure acetone, and the acetone quickly sucked away. This is repeated until the acetone comes through colorless, at which time the powder on the filter will also be colorless. The total volume of acetone extract will vary between 400 and 600 cc., depending on the kind of leaves used.

Removal of the chlorophyll. The green acetone extract is divided into parts of 100-200 cc., to each of which 200-250 cc. of ether are added and the acetone washed out with distilled water. The ether fractions are now combined, dried over anhydrous sodium sulfate and filtered through a dry filter into a 200 cc. graduated flask which is filled to the mark with dry ether.

One hundred cc. of this ether are saponified with 2 cc. of concentrated methyl alcohol solution of KOH by shaking carefully by hand and then in a shaking machine for 30 minutes. After standing a little while the ether is usually a pure yellow color, but if it still shows a red fluorescence the shaking is continued, if necessary with the addition of more alkali. After complete saponification of the chlorophyll the ether solution is decanted from the alkali-chlorophyllines into a small separatory funnel and the chlorophyll salts washed gently with ether. In order, however, to completely free the precipitate of occluded xanthophylls 30 cc. more ether are added to the alkaline material, the mixture shaken, and, after adding water, allowed to stand until the emulsion has broken. If necessary this is repeated with fresh ether.

The ethereal solutions thus obtained are washed with water to which a little methyl alcohol solution of KOH is added in order to separate traces of chlorophylline and small amounts of brown acid organic substances. The ether is finally washed twice with pure water and evaporated to a volume of a few cubic centimeters in a vacuum distillation flask at room temperature.

Separation of the carotinoids. The concentrated ether solution of carotinoids in the vacuum distillation flask is washed into a separatory funnel with 80 cc. of petroleum ether, the flask being washed out finally with a little ether. This solution is now mixed successively with 100 cc. of 85 per cent methyl alcohol, 100 cc. of 90 per cent

methyl alcohol and twice with 50 cc. of 92 per cent methyl alcohol. The last extract is generally colorless; if not, another extraction is made with 92 per cent alcohol.

The methyl alcohol extracts contain the xanthophylls. They are also free from carotin, according to Willstätter and Stoll. The combined methyl alcohol extracts are now mixed with 130 cc. of ether and the pigments transferred to the ether by a slow addition of water. The ether solution of the xanthophylls thus obtained and the petroleum ether solution of carotin are freed from methyl alcohol by washing twice with water. The solutions are then filtered through dry filters into 100 cc. graduated flasks, the solutions cleared up by the addition of a few drops of absolute alcohol and the flasks filled to the mark with ether and petroleum ether respectively.

Colorimetric comparison with standard solutions. The carotin and xanthophyll fractions, representing 20 grams of fresh leaves, are now ready for comparison with standard solutions in a colorimeter. For this purpose one can use either pure carotin or xanthophyll solutions in petroleum ether and ether, respectively, or their color equivalents, namely, 0.25 per cent alazirin in chloroform, or a 0.2 per cent aqueous solution of $K_2Cr_2O_7$. The pure pigment standards are not satisfactory because of their instability, the xanthophyll standard, especially, fading quite rapidly. The dichromate solution is especially well suited for a substitute because a standard solution once made will keep indefinitely. It is necessary, however, to know its color value in terms of the pure carotinoid pigment solutions. Using 5×10^{-5} molar solutions of carotin and xanthophyll, respectively, equivalent to 0.0268 per cent carotin solution and 0.0284 per cent xanthophyll solution, Willstätter and Stoll found the following relations to exist between the standard 0.2 per cent $K_2Cr_2O_7$ solution and the carotinoids.

100	mm.	carotin	solution	equals	101	mm.	$K_2Cr_2O_7$ solution
50	"	"	"	"	41	"	" "
25	"	"	"	"	19	"	" "
100	"	xanthophyll	"	"	72	"	" "
50	"	"	"	"	27	"	" "
25	"	"	"	"	14	"	" "

In Willstätter and Stoll experiments the standard carotinoid solutions only were apparently used. The standard solutions were always set at a depth of 100 mm. and the height of the unknown adjusted until the colors matched. Readings were then taken with the cups reversed in the colorimeter and the results averaged. A Wolff colorimeter was used by these investigators but a Dubosque or Kober

colorimeter should serve the purpose just as well. The writer has found the Kober colorimeter very satisfactory, using daylight as the source of illumination and the black glass cups with the colorless, optical glass bottoms for holding the solutions.

Calling h_c the height of the unknown solution required to match the color of 100 mm. of standard carotin solution and h_x the height of the unknown solution required to match the color of 100 mm. of standard xanthophyll solution the amount of carotin or xanthophyll in 1 kg. of fresh leaves can be calculated from the amount obtained from 20 grams by the method of Willstätter and Stoll by means of the following formulae:

$$\text{Carotin equals } 50 \times 0.00536 \times \frac{1}{2} \times \frac{100}{h_c}, \text{ and}$$

$$\text{Xanthophyll equals } 50 \times 0.00568 \times \frac{1}{2} \times \frac{100}{h_x}.$$

If the standard potassium dichromate solutions have been used in place of the pure carotin and xanthophyll, the same formulae are used because the dichromate is used at a depth of color corresponding to 100 mm. of the carotinoid solutions. These need not necessarily be set at the values corresponding to 100 mm. of the carotinoid solutions, but, if desired, can be set at the values corresponding to 50 or 25 mm. of the standard carotinoid solutions. In fact, the writer believes that more accurate determinations are secured by averaging the results obtained with the standards set at the equivalents of 100, 50 and 25 mm. of pure carotinoid solutions.

Results by Willstätter and Stoll's method. Table 19 shows some of the results obtained by Willstätter and Stoll using their own method. The data are averages of duplicate determinations reported in full by these investigators and show the difference between the carotin and xanthophyll content of leaves exposed to the light and those which are heavily shaded, both being obtained from the same plant. The fresh leaves which were in the shadow were in some cases appreciably lower in carotinoids than the leaves exposed to the light, but this difference appears to be due, in part, to a higher moisture content in the fresh shaded leaves.

The quantitative results of Willstätter and Stoll show a very different proportion between carotin and xanthophylls than was obtained by Monteverde and Lubimenko, which can not be due entirely to the fact that different plants were used in the two studies. The

TABLE 19. CAROTIN AND XANTHOPHYLL CONTENT OF LEAVES (METHOD OF WILLSTÄTTER AND STOLL)

Name of plant	Condition	Pigment in fresh leaves: Carotin mg. per 100 gms.	Pigment in fresh leaves: Xanthophyll mg. per 100 gms.	Pigment in dry leaves: Carotin mg. per 100 gms.	Pigment in dry leaves: Xanthophyll mg. per 100 gms.
Sambucus nigra	Light-exposed	14.1	26.3	52.5	97.7
Sambucus nigra	Shaded	6.3	19.2	38.5	118.0
Aesculus hippocastanum	Light-exposed	29.3	45.1	79.0	121.0
Aesculus hippocastanum	Shaded	9.3	27.9	37.0	111.0
Platanus accrifolia	Light-exposed	12.9	27.8	38.0	82.5
Platanus accrifolia	Shaded	12.7	31.1	51.0	125.0
Fagus silvatica	Light-exposed	18.5	30.0	....	.....
Fagus silvatica	Shaded	13.1	25.2	35.0	68.0
Populus canadensis	Light-exposed	9.7	....	29.0	.....

writer is inclined to believe that the ratio of 1.5 to 2 molecules of xanthophyll to 1 of carotin, as found by Willstätter and Stoll, represents more nearly the true proportion between the two classes of carotinoids as they exist in green leaves.

Elizabeth Goerrig (1917) has applied the general principles of the Willstätter and Stoll method to the determination of the carotin and xanthophyll content of yellow autumn leaves. This work has already been discussed in Chapter II in connection with the pigments of autumn leaves, but it might be well to mention here Miss Goerrig's experience in applying the method. She varied the procedure in several particulars, one of the most important of which, as far as its possible effect on her results is concerned, was the preliminary drying of the leaves at 40° C., instead of using the fresh leaves as recommended in the original method. Miss Goerrig admits that the dried leaves were difficult to grind with the extraction solvent and, in fact, states that the yellow leaves usually retained a part of the color which could not be extracted by the method recommended. Moreover, the calculation of the carotin content of some of the leaves using Miss Goerrig's data, which are expressed as colorimeter readings only, gives results much lower than Willstätter and Stoll reported for leaves from the same species of plant. Another important particular in which Miss Goerrig modified the Willstätter procedure was the omission of the preliminary extraction with 30 per cent acetone and the use of 85 to 90 per cent acetone for the extraction of the pigments instead of the pure acetone recommended. Finally, Miss Goerrig used a 0.4 per cent $K_2Cr_2O_7$ solution as a standard in place of a 0.2 per cent solution. By setting the standard at 50 mm. a greater range of color in-

tensity was secured for the unknown color solutions. No attempt was made to calculate the results in terms of carotin and xanthophyll content of the leaves studied.

Miss Goerrig mentions one or two points of interest in connection with the remaining steps of the method, which were followed closely. In the removal of the chlorophyll by saponification the alkali-chlorophyllines did not retain the xanthophylls as mentioned by Willstätter. Again, in the final removal of the xanthophyll to ether before making up the solutions for the colorimetric reading, Miss Goerrig encountered the most difficult part of the whole method. Contrary to the statement of Willstätter and Stoll, she found it impossible to transfer all the xanthophylls to ether by the slow addition of water.

Estimation of Fucoxanthin

The fucoxanthin content of brown algæ can be determined by a colorimetric method devised by Willstätter and Page (1914). The details of the isolation of the pigment and its quantitative estimation are given by these investigators as follows.

The algæ are pressed dry between filter papers and ground to a fine meal. Except for *Laminaria*, for which a different treatment is recommended, 40 grams of the meal are mixed with 200 grams of sand and macerated with 50 cc. of 40 per cent acetone, then twice with 50 cc. of 30 per cent acetone. These extracts are discarded. The pigments are then extracted with pure acetone. *Laminaria* are first cut up into small pieces and extracted with 30 per cent acetone in a beaker. The pulp is then ground in a meat chopper and a weighed quantity mixed with sand and extracted with 95 per cent acetone and finally with anhydrous acetone until all the pigments are extracted.

In all cases the pigments are transferred to ether by adding 300 cc. of ether to the acetone solution and then adding distilled water. The ether is freed from acetone by very careful washing with distilled water and, after mixing with an equal volume of petroleum ether, is ready for the extraction of the fucoxanthin. This is accomplished by shaking four times with an equal volume of 70 per cent methyl alcohol which has been saturated with petroleum ether, the volume of the upper layer being kept constant by additions of ether after each extraction. The combined alcohol extracts are freed from some xanthophyll which is extracted along with the fucoxanthin by shaking

with an equal volume of a mixture of five parts petroleum ether and one part ether. Some fucoxanthin is lost in this extract but it is recovered by concentrating the extract to 250 cc. in vacuum, adding an equal volume of ether and extracting twice with 500 cc. of 70 per cent alcohol, which has been saturated with petroleum ether. The new alcohol extract is added to the first main extract. The fucoxanthin is finally transferred to ether, which is freed from methyl alcohol by washing with water, and made up to a volume of 250 cc. in a graduated flask.

The solution is now compared in a colorimeter with either a standard fucoxanthin solution (using a 5×10^{-5} molar, or 0.0304 per cent solution) or the 0.2 per cent $K_2Cr_2O_7$ standard which is used for estimating carotin and xanthophylls. The standard is set at 50 mm. of standard fucoxanthin or 85 mm. of the dichromate solution, which is its equivalent, and the depth of the unknown solution which is required to match the color determined. If the height of the unknown is h_f the content of fucoxanthin in 1 kg. of fresh algæ as calculated from the 40 gram sample will be $25 \times 0.00608 \times \frac{1}{2} \times \frac{50}{h_f}$, if the standard fucoxanthin solution is used, or a similar result if the dichromate has been used, since the latter will be set at the equivalent of 50 mm. of standard fucoxanthin.

Using this method Willstätter and Page determined the fucoxanthin content of *Fucus*, *Dictyota* and *Laminaria* to be, respectively, 169 mgs., 250 mgs., and 81 mgs. per kg. of fresh algæ.

Application to Other Biological Materials

It seems obvious that the colorimetric methods of analysis for carotin, xanthophylls and fucoxanthin as worked out by Willstätter and his co-workers should be applicable to any biological material containing these pigments if a suitable method can be devised for freeing the pigment from the tissues involved. It would seem that the Willstätter technic can be applied without modification to plant tissues, including flowers, fruits and leaves, with the exception of the fruits containing lycopin, for which no quantitative method has yet been devised. Moreover, the writer is not aware of any methods for separating carotin from lycopin so that even a quantitative estimation of

carotin is not possible in fruits in which these two carotinoids are present together.

For animal tissues and fluids containing only a single carotinoid an extraction with ether or petroleum ether either directly, if sufficiently dry, or after treatment with alcohol, if much water is present or if the pigment is bound to protein, should yield a solution which may be used at once for quantitative estimation, colorimetrically. A preliminary concentration of the extract, previous to comparing with the standard, may be advisable. For blood work the writer concentrates the extracts to the original volumes of blood taken (usually 10 cc.) so that the colorimeter readings can be calculated directly to the percentage of carotinoid in the blood. In the case of animal fats, like butter fat or adipose tissue fat, the approximate concentration of carotinoid present (assuming that only one is present) can be determined at once by comparing the rendered, melted fat with the standard in the colorimeter. If the fat is highly colored the necessity of keeping the fat melted can be avoided by diluting with an equal volume of ether, inasmuch as no great difficulties in the calculation of the results are thereby introduced.

For animal tissues and fluids containing both types of carotinoids in sufficient quantities so that the assumption of only a single type involves too great an error, it is not likely that a saponification of the extracts can be avoided because of the presence of more or less fat in nearly all animal tissues containing the chromolipoids. In carrying out this saponification and subsequent recovery of the pigments in the unsaponifiable matter, care should be taken to avoid the production of aldehyde resins pigments which might be caused by the use of impure alcohol. One to two cc. of 10 to 20 per cent alcoholic potash for each gram of material extracted for the pigment analysis would insure a large excess of alkali for the saponification and would keep the volume of fluids within the realm of easily conducted analyses. Following the saponification and extraction of the fat-free pigments from the soap, the combined pigments must be washed free from alkali and then concentrated to the lowest possible volume, preferably in vacuum, then diluted with petroleum ether of low boiling point and the pigments submitted to fractionation by the phase test between the petroleum ether and 80-90 per cent alcohol, preferably methyl alcohol. The separated carotin and xanthophylls can then be compared with the standard in the colorimeter, after diluting or concentrating to a suitable volume. In the case of xanthophyll pigments, it is well to first transfer to ether, as in the Willstätter technic for plant tissues.

Certain animal tissues whose carotinoid content may be desired may contain pigments soluble in alcohol or ether whose presence would interfere with the direct analysis of the extracts. Tissues such as liver, spleen, kidney, heart, etc. fall in this class. In most cases the foreign pigments can be removed by saponification, but this must be conducted with great care to avoid the production of other foreign pigments which may be extracted from the soap by ether and interfere, not only with the analysis, but also with the true demonstration of the presence of carotinoids. Bile pigments, if present, can usually be removed by treating the fresh tissues with lime water, previous to the extraction with ether, in order to form ether-insoluble calcium salts. It must be admitted, however, that the quantitative analysis of tissues of this character for carotinoids requires considerable study before it can be concluded that the method proposed is entirely free from error.

The final colorimetric comparison with the standard hardly needs further comment. It is obvious that the 0.2 per cent $K_2Cr_2O_7$ solution is the most convenient to use. For animal tissues, and perhaps some plant tissues, the amount of pigment present may be so low that a convenient quantity of tissue will not yield sufficient pigment to match the dichromate standard at any of the equivalent carotinoid depths given by Willstätter and Stoll. It is convenient in these cases to set the unknown solution at a given depth of say 50 mm. or 100 mm. and match its color with the standard dichromate. The question then arises as to the carotin or xanthophyll equivalent of the dichromate depth found in such an analysis. For this purpose the writer has constructed the curves shown in Chart 1. These curves are based on the somewhat meager data given by Willstätter and Stoll for the comparative color of 5×10^{-5} molar carotin and xanthophyll solutions with the standard dichromate solution and also the comparative color of the two pigment solutions.

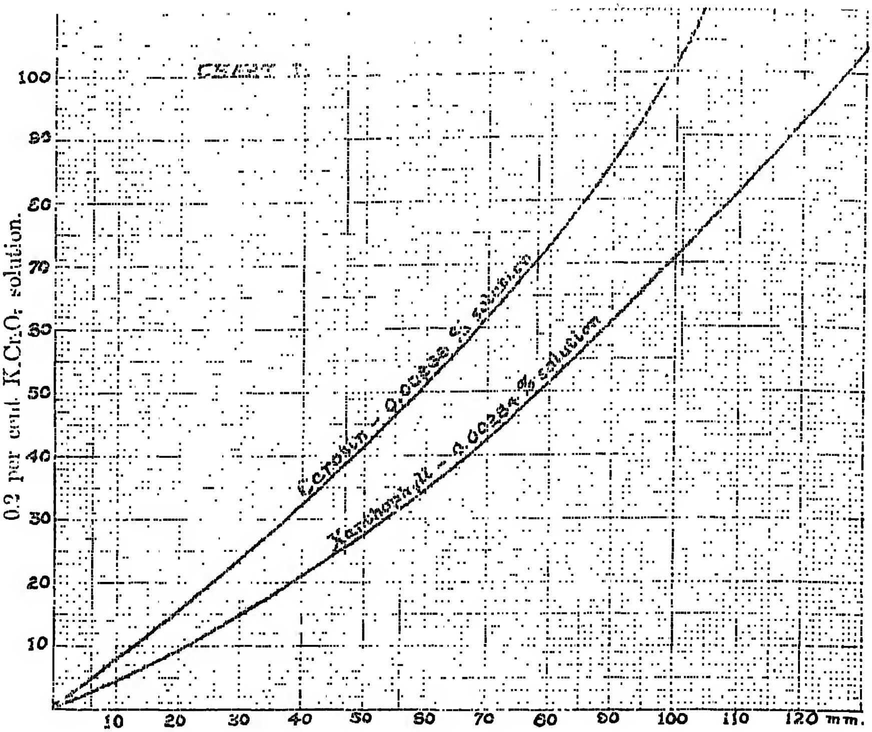
The method of using these curves involves no difficulties, but for the sake of clearness one or two examples may be given.

Example 1. A 25 mm. layer of melted butter fat from cows on fresh pasture grass was found to require 36.9 mm. of 0.2 per cent $K_2Cr_2O_7$ in the Kober colorimeter. Referring to the carotin curve in Chart 1 it is seen that 36.9 mm. of standard dichromate equals 45.2 mm. of 0.00268 per cent carotin solution.

Therefore, $0.00268 : x = 25 : 45.2$

$x = 0.00485$ per cent carotin in the butter fat (ignoring the sp. g. of the fat).

Example 2. The extract of 10 cc. of blood serum in 10 cc. volume in ether was compared colorimetrically with standard dichromate. A 50 mm. layer of serum extract was found to equal 15 mm. of 0.2 per cent $K_2Cr_2O_7$. The pigment eventually proved to be entirely xantho-



CAROTIN OR XANTHOPHYLL

Quantitative relations between 0.2 per cent $K_2Cr_2O_7$ solutions and 5×10^{-5} molar solutions of carotin and xanthophyll.

phyll. Referring to the xanthophyll curve in Chart 1 it is seen that 15 mm. of dichromate equals 30.5 mm. of 0.00284 per cent xanthophyll solution.

Therefore, $0.00284 : x = 50 : 30.5$

$x = 0.00173$ per cent xanthophyll in the serum (ignoring the sp. g. of the serum).

Summary

The quantitative estimation of carotinoids in plant and animal tissues must be carried out by colorimetric methods. Standard 0.2 per cent potassium dichromate solution serves for the color comparison with carotin, xanthophyll and fucoxanthin solutions. The quantitative relations between the standard and the pigments mentioned are described and charted in this chapter. The methods are also described in detail for preparing plant and animal tissues for the analysis as well as the separation of the individual carotinoids mentioned when present together in the plant extracts. Data are given showing the results of applying the methods to various plant and animal tissues.

No method has yet been devised for estimating lycopin quantitatively. A suitable standard must first be found and a method discovered for separating lycopin from carotin.

Chapter XI

Function of Carotinoids in Plants and Animals

The significance of the chromolipoids in the metabolism of the living organisms in which they are found has not been discovered. Their almost universal occurrence in vegetative organisms and their very frequent appearance in animals naturally leads to the belief that they perform some function in the economy of life. It is natural for the biochemist to seek for the basis of the occurrence of any wide-spread substance or group of substances in living matter but so far as the carotinoids are concerned their significance and possible functions have not got beyond the realms of speculation. In presenting the theories which have been advanced it seems best to consider the plant side of the question separately from that of the animal. There are several reasons for this. In the first place the carotinoids have their origin in the plants and occur in animals only as they are present in the food. In fact, as already pointed out in a previous chapter, the ability to synthesize the carotinoids may well serve as one of the means of distinguishing plants from animals. In the second place the question has recently been raised as to whether the carotinoid pigments are identical with vitamin A, or related to these unknowns which play so important a part in the nutrition of animals. This question is properly considered in connection with the possible function of the carotinoids in animal life. Finally, the occurrence of carotinoids in certain species of animals, such as fowls and cattle, has come to have a practical significance in connection with the use to which man has put these animals in the production of eggs and milk.

Before discussing these questions, some of which have a practical as well as a biochemical point of view, it is not altogether unreasonable to ask why it is necessary to consider that the carotinoids must play a rôle in metabolism merely because they are of wide-spread occurrence. It is not a new idea that the lipochromes in animals are of the nature of waste products of the organism. Although this idea was advanced while the belief was generally held that animals synthesize

their own lipochromes, the fact that animals merely derive their lipochromes preformed from their food does not invalidate the idea that these pigments are merely casual products in the animal organism and even suggests that they perform no useful purpose in the plants which synthesize them.

Possible Function in Plants

Various theories have been advanced to explain the significance of the carotinoids in plants. When Arnaud (1885) discovered the presence of carotin in green leaves he raised the question as to its possible relation to chlorophyll and later (1889) suggested that the pigment might play a rôle in plants similar to that of the hemoglobin of the blood. He also attached considerable significance to the fact that carotin, with its great affinity for oxygen when released from the living plant tissues, remains apparently unaltered at an almost constant level when in the living leaf. Arnaud could not explain this except on the basis that the carotin was constantly undergoing an alternating oxidation and reduction analogous to that of hemoglobin in the blood.

Zopf looked upon the lipochromes as reserve products, but Miss Newbigin (1898) has aptly stated that it is safer to admit merely that they often occur in association with reserves. Zopf, however, apparently limited his conception of carotin as a reserve substance to certain fungi, such as the *Uridineæ* (rusts) and certain molds. The idea was based upon his observation that the pigment seems to concentrate in the spores of these plants and later to disappear during germination. Kohl (1902j) accepted this idea and in addition stated that he believed that carotin acted as a reserve substance in the carrot root.

Kohl has, in fact, given us the most comprehensive conception of the various rôles which carotin may play in plant life. Primarily he believes with Engelmann (1887) that carotin shares with chlorophyll the work of carbon dioxide assimilation, and that this lies chiefly in its energetic absorption of a large part of the blue-violet rays of sunlight. This light is transformed into heat, a property which Stahl (1896) believed anthocyanin and carotin shared, permitting the pigment to act indirectly as a catalyst for various metabolic processes, including the decomposition of the atmospheric carbon dioxide. There can be no doubt that the spectroscopic properties of the carotinoids are one of the strongest arguments in favor of the view that they perform some definite function in the plant. Whether the light absorbed is transformed as Kohl believes or whether it serves some other purpose

is difficult to determine. Kohl (1906b) offered as proof of his theory the fact that etiolated leaves which would not turn green in partial vacuum did so when the partial vacuum was replaced by oxygen-free CO_2. He believed that this experiment shows that the carotin in the etiolated leaf is able to transform the CO_2 into oxygen for the formation of chlorophyll.

The spectroscopic absorption properties of the carotinoids may serve the purpose of protecting the cell enzymes against the destructive action of certain light rays, according to Went (1904). This theory has been supported by Kohl (1906c) also, who states that he was able to establish experimentally that carotin solutions exert a protective action towards diastase through their absorption of the violet and ultra-violet rays beginning about 420μμ.

With reference to carotin acting in a respiratory rôle through its power to absorb oxygen, as suggested by Arnaud, Kohl (1900j) thinks that it may so act in the chloroplastids, but that no general respiratory rôle can be ascribed to it inasmuch as respiration is known to proceed just as normally in colorless cells as in those which are pigmented. From a biological point of view Kohl believes that carotin shares with other pigments of flowers and fruits the function of an effective lure for insects and birds and other animals, in connection with the spreading of seeds and pollen.

Willstätter and Mieg (1907) agree with Arnaud that the most likely functions of the carotinoids are related to their great affinity for oxygen. They are inclined to regard the work of these pigments in the light of oxygen transference, however, rather than as directly concerned with the oxygen assimilation. They accept the possibility of a certain amount of oxygen absorption. They are careful to point out, however, that xanthophyll cannot well be the end product of this oxygen absorption; but that this product is probably concerned with the regulation of the oxygen pressure in the plant cells. With reference to a possible part which the carotinoids might play in carbon dioxide assimilation Willstätter and Stoll express the belief that an experimental demonstration is needed of the possibility of the carotinoids acting in such a rôle alone, inasmuch as it is a chemical function difficult to understand for these pigments. It would appear that Kohl (1906c) has already furnished a certain amount of evidence along this line, but this does not seem to be generally accepted by the plant physiologists. In fact, Miss Irving (1910) has shown that the greening of etiolated shoots is not indicative of the power of carbon assimila-

tion, but that this ability is still lacking even in such shoots which have developed a considerable amount of green color.

A somewhat different aspect to the possible function of the carotinoids in the chloroplastids is given by another theory of Willstätter and Stoll in which it is supposed that the carbon dioxide assimilation is controlled by the equilibrium between the chlorophyll components *a* and *b*, and that this equilibrium is in turn controlled by the carotinoids. The process, as imagined by Willstätter, is as follows. Carbon dioxide is attracted by the affinity of the magnesium compounds (chlorophylls) for CO_2, and is at once reduced by chlorophyll *a*. Chlorophyll *a* is thereby oxidized to chlorophyll *b*. Carotin then withdraws the oxygen from chlorophyll *b*, reducing it again to chlorophyll *a*, the carotin at the same time being oxidized to xanthophyll. The reduction of the xanthophyll to carotin, in order to complete the cycle of Willstätter's theory, is effected by a reductase. One difficulty with this theory is that Willstätter himself, as he has pointed out, does not admit that carotin can be oxidized to xanthophyll. The ability of carotin to reduce chlorophyll *b* and thereby become oxidized is unquestioned, as evidenced by its strong reducing action on ferric salts. Xanthophylls, however, share this property with carotin. At the same time some support is given to the theory of a functional relation between the chlorophylls and carotinoids by the possibility that fucoxanthin plays the part of chlorophyll *b* in the brown algæ, which lack this chlorophyll component.

Ewart has recently (1915) attempted to show that carotin and xanthophyll can play a part in photo-synthesis. His experiments purport to show that carotin yields HCHO when submitted to photo-oxidation in a stream of pure oxygen and that xanthophyll yields both HCHO and sugar under similar conditions. In view of the fact that Ewart's conception of xanthophyll includes the idea that it "is soluble in water and in any mixture of alcohol and water," and also since there is no assurance that his carotin was free from impurities, his results can not be given unqualified acceptance. In the same paper Ewart claims to have produced xanthophyll from chlorophyll, but Jörgensen and Kidd (1917) have shown that the "xanthophyll" which Ewart produced in his experiments was probably phaeophytin.

The question of the origin of the carotinoids in plants, which is suggested by Ewart's attempt to produce xanthophyll from chlorophyll, is closely related to the question of their function. The fact that the carotinoids form in etiolated plants without chlorophyll is a strong

point in favor of their independent existence, but does not, of course, show that the chlorophylls and carotinoids do not arise from a common nucleus. From a chemical point of view the most likely substance which could thus give rise to both carotinoids and chlorophyll would be isoprene, C_5H_8, the terpene "baustein" which may go to phytol on the one hand, as Willstätter believes, and perhaps could also go to carotin on the other.

Very little study has been given to the physiological conditions which govern the formation of the carotinoids in plants or to the problem of the relations between the different carotinoids or between the carotinoids and other plant constituents. The Toblers (1912) observed that the carotin content of carrots increased during the formation of starch from sugar, but it is difficult to decide from this observation that the carotin plays any part in the process. These investigators (1910b, 1912) have also shown that the formation of carotin and lycopin in the ripening of tomato fruits is coincident with the destruction of chlorophyll. Lubimenko (1914a) has concluded that the lycopin forms in this case at the expense of the chlorophyll, but there is no chemical basis for assuming that this indicates that the carotinoids are actually formed from the chlorophyll.

Duggar (1913) made an especially interesting study of the development of carotinoids in tomato fruits. He found that the factor for carotin formation and the factor for lycopersicin (lycopin) formation are present together in the fruits which normally redden on ripening, but that the formation of the red carotinoid could be partially or completely suppressed by ripening the green fruits at a temperature of 30° C. or above. At these temperatures the fruits ripened with a yellow color and contained only carotin and xanthophylls. The inhibition of the lycopin was found to be proportional to the temperature (between 30° and 37° C.), but was inversely related to the age of the fruits, the oldest fruits requiring a higher temperature for the suppression of reddening. The failure of the fruits to develop lycopersicin at the higher temperatures was found to be a true suppression, inasmuch as the red pigment formed rapidly when the yellow fruits were returned to a lower temperature. Duggar was also able to show very satisfactorily both by means of the microscope and the spectroscope that the lycopin which formed in these cases could not have been derived from the carotin present.

Duggar also made a study of other factors entering into the formation of the lycopin of tomatoes, with the result that the synthesis of

the pigment was found to be independent of light, but dependent upon oxygen. The fruits failed to redden in all cases of oxygen exclusion even at a favorable temperature, but assumed a greenish yellow, yellow or yellow-orange color with an accompanying loss of chlorophyll. Whether the latter colors were due to a formation of carotin and xanthophyll in the atmospheres of hydrogen and nitrogen employed, or whether the carotinoids were merely revealed by the destruction of the chlorophyll is not clear. Observations were also made on the catalase activity of the fruits as well as their titratable acidity under the conditions of lycopin suppression, with the result that it was found that a very low catalase activity and decreased acidity accompany the conditions which suppress the formation of the pigment. It is apparent, however, that these are not the only factors concerned.

Possible Function in Animals

A quarter of a century ago the majority of the biologists accepted the idea that all the visible pigments of animals, including the lipochromes, are essential products of the animal metabolism. The prevailing theories of evolution looked upon animal colorations as factors in the existence of the species which had persisted solely for some useful purpose. The theories which described the function of the pigments in various terms, such as Protective Coloration, Warning Coloration, Mimicry, Sexual Attraction, etc., all had their followers. It is not our purpose to discuss these theories. One can find them both adequately defended and impartially criticized in the treatises current during the closing years of the past century. Suffice it to say that the writer does not possess a biological viewpoint which is sufficiently developed along academic lines to appreciate "function" as an abstract attribute of living organisms. Function, to be real, according to his conception, must be concrete or physiological. Perhaps there are those who will regard this as going from one extreme to the other. If so, the explanation lies in the fact that this monograph deals only with animal pigments which are derived from the food. Such pigments, if possessing a function, must be linked with the physiological, in this case the nutritional or metabolic processes of the body.

We have seen in Chapter VII that great variation exists among different species of mammals with respect to their ability to absorb the carotinoids from their food and deposit the pigments in their tissues, or, if one prefers the opposite point of view, to destroy the carotinoids

ingested and thus prevent their deposition in the tissues. This fact alone seems to argue against the carotinoids performing any general physiological function in animals; *a priori*, a substance of general value in nutrition would most likely have a general occurrence and would at least always be present in the animal body. The complete absence of carotinoids from the tissues and secretions of certain species of animals and their almost complete absence from others, even on diets rich in carotinoid pigments, furnishes a sufficient basis, at least from a teleological standpoint, for rejecting any theory that the carotinoids exert a physiological function in animal life.

The writer became interested in this question from an experimental point of view in 1912, when it was found, in connection with the biological origin of the lipochromes of cattle, that the new-born calf of a highly pigmented breed of cattle showed an almost complete absence of carotinoids. This suggested the idea of raising animals to maturity on carotinoid-free diets, particularly those species which normally deposit the carotinoids in their tissues. Later, after the writer (1915) had shown that the lipochromes of fowls are derived from plant xanthophyll, plans were laid for carrying out such an experiment on these animals, because of the obvious advantages associated with a smaller, more rapidly growing species.

The problem was primarily one of selecting a ration entirely devoid of carotinoids, particularly xanthophyll, but otherwise adequate for normal growth. The problem had the added interest that the rapidly growing subject of vitamins had already (1916) indicated a casual relationship between the occurrence of fat-soluble vitamin A and carotinoids in certain foods such as butter and green leaves, and the absence of both substances from lard. The experiments showing the possibility of raising fowls on diets lacking the natural pigment of their adipose tissue, which were begun in 1916 and were reported by Palmer and Kempster (1919a) were not designed, however, to show the relation between carotinoids and vitamin A. The writer dismissed the possibility of any such relation as the result of the experiment carried out in the winter of 1916-17 in which young chickens weighing 700 to 750 grams were raised to maturity and exhibited normal fecundity on carotinoid-free rations. The successful termination of a later experiment in this series, in which a flock of 50 chickens was raised from hatching to maturity on similar diets, showed conclusively for the first time that a species of animal which is normally pigmented with

carotinoids does not require these pigments for its growth or for the reproduction of its kind.

While the last experiment was in progress Drummond (1919) reported the failure of pure crystalline carotin, fed at the rate of 0.003 per cent of the ration to improve the condition of albino rats suffering from vitamin A deficiency; while Steenbock, Boutwell and Kent (1919), on the other hand, were calling attention to certain new associations of yellow pigmentation and vitamin A and were suggesting that the two were at least associated in some way. Although the statement was made that vitamin A "is not carotin," this was later retracted by Steenbock (1919) and the provisional assumption advanced that this vitamin is one of the carotinoid pigments. In support of this assumption Steenbock and his associates have published a series of papers showing that a rather close correlation exists between carotinoid pigmentation and vitamin A content of roots (Steenbock and Gross, 1919; Steenbock and Sell, 1922), maize (Steenbock and Boutwell, 1919a), leaves (Steenbock and Gross, 1920), and peas (Steenbock, Sell and Boutwell, 1921), as determined by feeding pigmented and colorless varieties of these plant products to albino rats. However, in studying the extractability of vitamin A from carrots, alfalfa, and yellow maize by fat solvents, Steenbock and Boutwell's (1920b) results show that highly colored extracts [1] do not exhibit the vitamin activity which would be expected if vitamin A is a carotinoid; and yellow maize, even after extraction with hot ether is shown to have lost very little vitamin A, although there must have been a considerable loss of pigment. On the other hand, especially favorable to Steenbock's theory was the finding in this paper that the fat-soluble vitamin follows the carotin in the application of the phase test to the unsaponifiable extracts from alfalfa leaves. When one bears in mind, however, that the solvents employed in the separation of the carotinoids by this method are respectively exceedingly poor and very excellent fat solvents, it is not surprising that the fat-soluble vitamin will follow the substance which goes into the better fat solvent.

When Steenbock, Sell and Buell (1921) attempted to obtain support for Steenbock's theory among animal products the correlation between pigmentation and vitamin content was so poor when comparing practically colorless codliver oil with butter fats of high and low color that

[1] Drummond and Zilva (1922) have substantiated this. They find that only "very slight" growth in rats is promoted by as much as 2 grams daily of the crude oil extracted from yellow maize by petroleum ether. This is a high proportion of the diet of young rats.

Steenbock has been forced to abandon his position that the two substances may be identical and to admit that their "coincident occurrence in nature might be due to physiological determination, pure and simple." The attempts to show some correlation between the color of perinephritic beef fat and vitamin A content in the same paper are not especially convincing on close examination, especially when the results are compared with butter fat of like color fed at much lower levels. In addition, the statement is made that egg yolks of a light color but with a normal vitamin content can be produced on specially selected rations, which confirms the observations of Palmer and Kempster (1919b) and Palmer and Kennedy (1921).

The lack of correlation between pigmentation and vitamin content of animal fats was first pointed out by Drummond and Coward[2] (1920) for butter fat and a large number of other fats and oils (including vegetable oils). It is of interest that colorless dog fat and colorless perinephritic pig fat were relatively rich in vitamin A. Miss Stephenson (1920) further corroborated this by decolorizing butter fat with charcoal without impairing in any way its vitamin content. This experiment, however, requires confirmation, primarily because of the remarkably small amount of charcoal which was used. As stated in Chapter IX, the writer has not yet succeeded in duplicating these decolorizations with only 2.5 per cent of any decolorizing carbon which he has been able to secure.

Further proof that vitamin A is not necessarily associated with carotinoids was furnished by Palmer and Kennedy (1921) who found that albino rats grew normally and reproduced on diets in which practically carotinoid-free ewe milk fat (containing 0.00014 per cent carotin) furnished the vitamin A in the ration at levels of 5 to 9 per cent, and that similar results followed the use of carotinoid-free egg yolk produced by hens on diets made up of selected white corn, skim milk, pork liver (about 10 per cent) and grit. With the rations containing carotinoids, the best results were secured with only 0.126 parts of carotinoid per million of ration. This is very much less pigment than Drummond or Miss Stephenson fed to rats without success. In opposition to this result Steenbock, Sell, Nelson and Buell (1920) have

[2] Previous to this, Rosenheim and Drummond (1920) were much attracted by the idea of an intimate relationship between carotinoids and vitamin A, and abandoned it very reluctantly when they were unable to establish an identity of vitamin A with either carotin or xanthophyll. Van den Bergh, Muller and Broekmeyer (1920) have also supported Steenbock's theory, without, however, submitting it to experimental verification.

stated that "carotin of constant melting point through a number of crystallizations was always found to induce growth in rats after growth had been suspended by a lack of fat-soluble vitamin in the diet." It is not stated how much carotin was fed. The statement is followed, however, by the naïve assertion that, "in spite of this it is not meant to infer that the fat-soluble vitamin is necessarily a pigment." In the same note, Steenbock and his associates state that they have prepared crystalline acetyl derivatives of constituents in the non-saponifiable vitamin fraction of the extracts from alfalfa hay, without resultant destruction of the vitamin. This fact alone is incompatible with a carotinoid nature for vitamin A. These pigments being hydrocarbons or hydrocarbons with an ether-like nucleus are quite incapable of forming acetyl derivatives.

Some light on the cause of the coincident occurrence of vitamin A and carotinoids is furnished by the recent experiments of Coward and Drummond (1921) who find that the synthesis of vitamin A is associated with the formation of chlorophyll. Their results showing the presence of little if any fat-soluble vitamin in etiolated seedlings and red sea-weeds, which are certainly not wanting in carotinoids, but which lack chlorophyll, support our own conclusion that vitamin A and carotinoids are not necessarily associated. The finding is of added interest because it shows that examples of this lack of association occur in the vegetable world as well as in the animal kingdom.

These results when considered together with the results of Drummond and Steenbock, as well as those of Palmer and Kennedy, showing the lack of definite correlation between the carotin and vitamin content of milk fat, indicate very clearly that the animals which transfer carotin abundantly from the food to the milk, as well as those which do not do so, have the power to separate pigment and vitamin. The writer suggests that the presence of appreciable amounts of vitamin A in the almost colorless butter fat examined by Steenbock may have come from more or less yellow maize in the diet of the cows. Palmer and Eckles (1914a) found that yellow maize has no appreciable effect on the color of butter fat, but it should bolster up the vitamin content of the butter, according to Steenbock's findings on the relative vitamin content of yellow and white maize. In an analogous manner it should be possible to produce eggs with low pigmented yolks, high in vitamin A, by limiting the pigmented part of the hen's ration to carrots, for the writer (1915) has shown that the feeding of carrots has little influence on the color of the yolks of hen's eggs.

An association of carotinoids with other vitamins than vitamin A and with the results of other dietetic deficiencies has also been suggested. Wiehuizen and others (1919) called attention to the low lipochrome content of the blood serum in the case of human beriberi and inferred a relationship between lipochromes and the antineuritic vitamin by stating that animal and vegetable substances with a high lipochrome content also have a high anti-beriberi value. It hardly seems possible that anyone with a thorough knowledge of the distribution and properties of vitamin B could give this suggestion any serious thought.

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A somewhat different conception of the significance of carotinoids in nutrition is presented by McCarrison (1920) who noted that butter made from milk of cows on green feed, and therefore high in pigment, afforded greater protection against edema of the adrenals of pigeons fed on autoclaved rice than less highly colored butter fat made from milk of cows on dry feed. McCarrison suggests that the hypothetical anti-edema substance may be of the nature of a lipochrome, but his results can also be explained on the basis of the seasonal (dietary) variation in the vitamin A content of butter.

We see from the foregoing discussion that there is little evidence to support the idea that the carotinoids exert a definite physiological function either in the species of animals in which they are visible after absorption or in those animals which do not appear to absorb the pigments at all. Curiously enough, however, a very practical use has been made of the appearance of carotinoid pigments in certain of the visible skin parts of some species which absorb the pigments. One may perhaps be justified in discussing these uses briefly in connection with the possible function of the pigments, although the function in these cases is in the service of man.

Practical poultry men in this country have recognized for a number of years that a relation exists between the amount of yellow pigment in the shanks, ear lobes, beaks, etc., of hens of certain breeds of poultry, such as Leghorns, Plymouth Rocks, Wyandottes, and Rhode Island Reds, and their previous egg laying activity. When Blakeslee and Warner (1915a,b) and Blakeslee, Harris, Warner and Kirkpatrick (1917) made extensive biometric analyses of data collected to determine the character and extent of this relation it was found that a definite positive correlation existed between pale shanks, ear lobes, beak, etc., and a recent more or less large egg production. As the result of these studies American poultry experts have made extensive use of the

appearance of the normally colored skin parts of these breeds of poultry at the end of the laying season for the purpose of culling out the unprofitable hens. This method of determining heavy from light laying fowls is not applicable, of course, to the breeds of poultry, such as the English Orpingtons, which never show yellow pigment in the visible skin parts, although normally their adipose tissue and egg yolks are colored with xanthophyll.

Palmer and Kempster (1919b) made a study of the physiological cause of the fading of the visible skin parts during egg laying. The ascertaining of the correct cause of this phenomenon was made possible by the success which we had in raising a flock of pigmentless White Leghorn fowls to maturity, the females showing normal egg laying activity. It was found that xanthophyll appeared in the skin of non-laying fowls within a few days[3] after feeding xanthophyll-containing foods, but that no pigment whatever appeared in the skin, and almost none in the adipose tissue of the hens which were laying, although only moderately (two eggs or less a week), *even after a month on xanthophyll-rich diets.* The blood serum and egg yolks contained an abundance of xanthophyll. It was also found that when pigmented fowls which were not laying (in these cases cockerels were used) were placed on carotinoid-free diets, they gradually lost the pigment from the visible skin parts in the same manner as laying hens. Histological studies of the skin during this fading indicated that the movement of the pigment was outward from the rete of Malphigi, where it is chiefly localized, towards the epidermis. No evidence was obtained that the loss of pigment was due to resorption but the indications were rather of a normal replacement of epidermis cells by the columnar pigmented cells of the Malphigian layer from beneath which carried less and less pigment because the supply of pigment in the food had been cut off. The fading of a highly pigmented skin is very gradual and usually requires several months in the absence of carotinoid from the food or in the case of egg laying.

The collective data were interpreted to mean that the fading of the skin during egg laying is the result of the deflection of the xanthophyll of the food to the ovaries, resulting in a cutting off of the pigment which would otherwise be excreted by the skin, the net result being the same as if the xanthophyll was no longer being ingested in the food. The writer believes that a continuous formation of ova, but not neces-

[3] In one case the color was distinctly visible in 72 hours after xanthophyll was introduced into the ration.

sarily with great frequency, is required to prevent the excretion of xanthophyll by way of the skin, and thus brings about a gradual fading of the visible skin parts. Whether there is a mobilization of pigment in other organs of the body, such as the liver, was not determined in our experiments.

Rosenheim and Drummond (1920) have expressed the view that this deflection of xanthophyll to the ovaries during egg laying indicates that the pigment is required for a definite and important function in the egg and that this fact thus supports the theory that the carotinoids are related to the vitamins. It is just as reasonable to suppose, however, that the egg yolk is an easier path of excretion for a fat-soluble pigment than is the skin, just as the kidneys are ordinarily the chief path of excretion of water-soluble waste products. Nevertheless, it might be worth while to investigate the relation between this whole phenomenon and the more recent interpretation of the effect of Nile blue on the pigment granules in the epidermis of the chicken skin, namely, that the pigment is transported there in association with fatty acids. It is possible that the concentration of the fat synthesizing powers of the hen in the ovaries during egg laying prevents the secretion of fatty acids by the blood capillaries and thus causes a concentration of xanthophyll in the fat laid down in the ova. This does not explain, however, why Sudan III, a fat dye, never appears in the skin when fed to either laying or non-laying fowls, although it appears abundantly in the egg yolk, bone marrow and adipose tissue, and feathers.

A phenomenon somewhat analogous to the fading of the skin of fowls during egg laying has been observed in the case of salmon during their fresh-water migration to the spawning beds from the sea, during which time the animals starve. As described by Miss Newbigin (1898), the flesh of the fish has the familiar strong pink color and the small ovaries a yellow-brown color when the fish come from the sea. As the reproductive organs develop the flesh becomes paler and the rapidly growing ovaries acquire a fine orange-red color. The explanation of this phenomenon unquestionably lies in the mobilization of the fat stores of the body in the reproductive organs and the shed ova, rather than in a mobilization of pigment itself. It is to be remembered that the fish are taking no food whatever during their migration, and must therefore draw upon every possible reserve, not only for their own needs but also for the reproduction processes for which the journey is taken. Essentially this view of the phenomenon was adopted by Miss Newbigin.

For three-quarters of a century the breeders of Guernsey cattle, one of the Channel Island dairy breeds, have laid great emphasis upon the fact that under comparable conditions the milk and butter from these cows has a higher yellow color than is produced by any of the other known breeds of dairy cattle. It is also generally recognized by the breeders of these cattle that a high yellow secretion by the skin is related to the production of highly colored milk and butter. These yellow secretions are usually localized at certain parts of the body, especially in the ear, on the end of the tail bone, and about the udder. In fact, at the present time the official scale of points for judging Guernsey cattle includes 15 points for skin color on the parts of the body mentioned. In judging bulls a similar allowance is made for high color in the ears and on the tail and body generally as indicating the ability of the animal to transmit the production of highly colored milk to the offspring. Jersey cattle show the same characteristics but not to so great an extent. It should be stated, however, that the ability of cows of the Guernsey breed to transfer the carotin from their feed to the milk is not so firmly fixed in the breed generally as the enthusiastic advocates of the breed would lead one to believe. Hill (1917) states that on the Island of Guernsey itself there is a marked difference between the color of the butter brought to market from different herds.

There is also a general feeling among the Jersey and Guernsey cattle breeders that abundant yellow secretions localized on the body indicate large producers of butter fat. Hooper (1921), who tried to correlate these ideas from observations which he made on about 160 animals, could find no relation between either the amount or color of the secretions and the production of either milk or butter fat, using yearly production records as the basis for his conclusions. The general idea is seen to be quite the reverse of the relations found to exist between the color of the skin of fowls and egg production. As a matter of fact if the phenomena of milk production, especially of milk fat production, and egg production are related physiologically the correlation between the production of milk fat and the color of the skin secretions should be between high production and low skin color and not between high production and highly pigmented skin as the breeders of Jersey and Guernsey cattle seem to think. Furthermore, by analogy with the hen, the fresh cow or the dry cow is not suited for judging the fat-producing ability by the amount or color of the skin secretions, but rather only the cow at the close of her lactation period. So far as the writer has been able to ascertain no observations have ever been

made indicating that the yellow skin secretions of Jersey and Guernsey cattle change their appearance with advance in the lactation period. It is perhaps not too hazardous to predict that it is only along such lines that a correlation may be expected to exist between skin pigmentation and butter fat production for cows of the Channel Island breeds. As a matter of fact Hooper's data show a slight indication of such a correlation for one group of cows but not for the other whose records and skin colorings are recorded. An investigation of the theory from the point of view of a fading of the skin color during heavy production might lead to very profitable results.

This brief discussion indicates the practical ends which may be served through the occurrence of plant carotinoids in the animal body. The whole subject is a fascinating one and offers as many unsolved problems as any other phase of experimental biology and biochemistry. The writer can not conclude this monograph, however, without inviting the attention of the biochemists to this field of work. The extension of the frontiers of our knowledge regarding these pigments which are so abundantly distributed in so many plants and animals is certain to prove a profitable as well as an interesting undertaking. Who can predict the magnitude of importance of the discovery which lies just beyond the horizon in this or any other expedition in the search after truth?

Summary

The functions of the carotinoids in plant tissues have not been definitely determined. The various theories which have been advanced include the following:

(1) Carotin plays a rôle in plants similar to that of the hemoglobin of the blood (Arnaud).

(2) Carotin acts as a reserve substance (Zopf, Kohl).

(3) Carotin shares in the work of CO_2 assimilation by acting indirectly as a catalyst for the decomposition of atmospheric CO_2 through its absorption of light energy which it helps to transform into heat (Kohl).

(4) Carotinoids protect cell enzymes against the light rays which they absorb (Went, Kohl).

(5) Carotin in flowers and fruits acts biologically as a lure for insects, birds and other animals, in connection with the spreading of pollen and seeds (Kohl).

(6) Carotinoids help regulate the oxygen pressure in plant cells through their great affinity for this element (Willstätter and Mieg).

(7) Carotinoids help control the CO_2 assimilation by controlling the equilibrium between chlorophyll a and chlorophyll b (Willstätter and Stoll).

(8) Carotin and xanthophylls play a part in photo-synthesis because they are believed to yield HCHO on photo-oxidation, xanthophyll also yielding sugar (Ewart).

There is no evidence to indicate that carotinoids originate from chlorophyll, but it is possible that both classes of pigment may arise from isoprene, C_5H_8.

The factor for lycopin formation in tomatoes can be suppressed at 30° C. or above, the fruits forming only carotin and xanthophylls. At lower temperatures all three types of carotinoids are formed. Synthesis of lycopin is independent of light but depends upon oxygen, and is depressed by the conditions which accompany low catalase activity and decreased acidity.

The author believes that if the carotinoid pigments in animals possess a definite function, this function must be linked with the physiological processes of the body, inasmuch as the carotinoids are derived from the food. There are a number of general facts, however, which indicate that these pigments play no definite rôle in nutrition or in metabolic processes, at least in the higher animals.

A critical review of the theories regarding the possible relation of carotinoids and vitamin A leads to the conclusion that the substances cannot be identical. It appears that there is a fairly definite correlation between the occurrence of carotinoids and vitamin A in plant tissues but not in animal tissues or in animal fats. Animals, therefore, possess the power to separate carotinoids and vitamin A. Experiments are suggested whereby this fact can be further substantiated.

Xanthophylls in fowls have a definite function from the standpoint of practical utility in that there is a correlation between low pigmentation of the visible skin parts of certain breeds of fowls and high egg production. The cause of this phenomenon is a selective mobilization of pigment in the ova during egg production, preventing its excretion by means of the skin. An analogous phenomenon occurs in the salmon during their fresh-water migration to the spawning beds.

It is generally believed by certain cattle breeders that abundant (carotinoid) pigmentation of the skin of Guernsey and Jersey cattle

is correlated with large fat production in the milk. The author suggests the possibility that if a correlation does exist in this case it is between low pigmentation and high production rather than the reverse, and is analogous to that which is found in laying hens.

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